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EDITOR-IN-CHIEF
C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR

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The Society of American Bacteriologists

THE FORTIETH GENERAL MEETING WILL BE HELD IN SAN FRANCISCO, CALIF.

August 30 to September 1, 1938

PROGRAM: A symposium on "Enzymes in Relation to Bacteriology" has been arranged for a joint meeting of all the sections of the Society.

The program for the Section on Agricultural and Industrial Bacteriology will include papers on nitrogen fixation and on the bacteriology of food, water and sewage.

The Section on Medical Bacteriology, Immunology and Comparative Pathology will hear groups of papers on the filterable viruses, rickettsial diseases, and various subjects of special significance in the western part of the United States.

Round-Table Discussions on the staphylococci, on the spirochetes, on the cross-inoculation of legumes, on the bacteriology of water and on the non-symbiotic fixation of nitrogen have been planned.

The program with titles of papers will be sent to all members in advance of the meeting. Abstracts of the papers will be distributed at the time of the meeting and published in the September number of the *Journal of Bacteriology*.

The Program Committee is composed of the following:

GEORGE P. BERRY, *Chairman of the Program Committee*, School of Medicine, University of Rochester, Rochester, N. Y.

EDWIN W. SCHULTZ, *Chairman of the Section on Medical Bacteriology, Immunology and Comparative Pathology*, Stanford University, Calif.

PAUL J. BEARD, *Chairman of the Section on Agricultural and Industrial Bacteriology*, Stanford University, Calif.

HEADQUARTERS: The Hotel Fairmont, San Francisco, Calif. Accommodations at special rates will be furnished to members of the Society. Reservations should be made with the hotel management.

EXHIBITS: Members desiring to give exhibits or demonstrations should arrange with Herbert G. Johnstone, University of California Medical School, Medical Center, San Francisco, Calif.

COLIFORM INTERMEDIATES IN HUMAN FECES

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Although foundation for the recognition of coliform intermediates was laid by Brown (1921), he did not utilize his findings for their demonstration. Brown was interested in mediums containing citrated blood and attempted to determine the effect citrate might have on the growth of organisms likely to be encountered in blood culture work. Koser (1923, 1924a, b, c, d, 1926) applied to coliform bacteria (Breed and Norton, 1937) tests involving utilization of organic acids and their salts. He found that *Bacterium coli* from feces could not utilize citrate as a sole carbon source in contrast to *Bacterium aerogenes*. Koser found, particularly in soil and water, organisms classed as coli by the criteria then used but which utilized citrate-carbon and, in this respect, resembled *B. aerogenes*. In 1924 Koser applied the term "intermediate" to these forms.

Recognition of such intermediate forms should make possible a better theoretical understanding of the entire coliform group, than which few in bacteriology are more complex or confused. Any fundamental advance, such as the intermediate concept, should enable sanitary science to define more accurately such terms as "pollution" and "potability" and to answer numerous practical questions posed in this field in recent years.

Parr (1936a, b, 1937) defined intermediates as coliform organisms "which have one or more coli characters and one or more of those attributed to aerogenes, and some 'intermediates,' including the typical fecal form, produce hydrogen sulphide." This definition permits the inclusion as intermediates of organisms

which are Voges-Proskauer positive, a feature we feel essential and justified by our data.

The three characteristics, defining coli and aerogenes, most emphasized in recent papers are the methyl-red and Voges-Proskauer reactions and the utilization of citrate. To these it is felt indol production should be added. Formerly, following Houston, a water was considered polluted if it gave a "lactose +, indol +" reaction and the "Flaginac" mnemonic of English sanitarians, defining the colon bacillus, analyzes into "FL," fluorescence in neutral-red broth; "AG," acid and gas in lactose broth; "IN," indol from tryptophane; and "AC," acidity with coagulation in milk.

Parr and Caldwell (1933a, b) did not include the Voges-Proskauer reaction, under the impression that this reaction shows perfect correlation with the methyl-red reaction (Levine, 1916) and for the sake of uniformity the bored latrine studies by Caldwell and Parr (1937) utilize the same classification. It is now known that many coliform organisms do not exhibit perfect correlation, that is, they are not necessarily methyl-red positive when Voges-Proskauer negative and vice versa. Hence, in the present project four tests, i.e., indol production, methyl-red and Voges-Proskauer reactions, and citrate utilization were used as fundamental differentials in the classification of coliform organisms into coli, intermediates and aerogenes.

To fix and facilitate expression of results we devised the mnemonic "Imvic," (Parr, 1936b). Thus *Bacterium coli* (*Escherichia coli*) is + + − −, which means that it is indol positive (I), methyl-red positive (M), Voges-Proskauer negative (V), and citrate negative (C). *Bacterium aerogenes* (*Aerobacter aerogenes*) is − − + +, which means that it is indol negative, methyl-red negative, Voges-Proskauer positive and citrate positive. Table 1, a compilation of the tests used by some of the workers in this field in the order in which they list the tests used, is evidence for the need of some such standardization and justification for the selection of the members of the Imvic quartet of tests.

The 16 types, which combinations of the four characters determine, are listed in table 2. In this table the types encountered by recent investigators are indicated.

TABLE 1

Summary of differential tests used in the study of coliform intermediates

Koser (1924)	M.R.	V.P.	Uric A.	Citrate
Kline (1930)	M.R.	V.P.	Uric A.	Citrate
Ruchhoft et al. (1931)	Indol	M.R.	V.P.	Citrate
Gray (1932)	M.R.	V.P.	Citrate	
Skinner and Brudnoy (1932)	Cello.	Citrate	Indol	Sucrose
	V.P.	M.R.		
Parr and Caldwell (1933)	M.R.	Indol	Citrate	
Bardsley (1934)	M.R.	V.P.	Indol	Uric A.
	Citrate			
Bigger (1934)	Indol	M.R.	V.P.	Citrate
Tittsler and Sandholzer (1935)	Cello.	Citrate	H_2S	A-M-D-G
Wilson et al. (1935)	M.R.	V.P.	Citrate	Indol
	Eijkman	Gelatin		
Raghavachari and Iyer (1935)	M.R.	V.P.	Indol	Citrate
Kline (1935)	M.R.	V.P.	Citrate	Indol
	Eijkman			
Griffiths and Fuller (1936)	V.P.	M.R.	Citrate	Uric A.
	Indol			
Parr (1936)	Indol	M.R.	V.P.	Citrate
Bartram and Black (1937)	M.R.	V.P.	Citrate	Indol
Hook and Hitchener (1937)	M.R.	V.P.	Indol	Citrate
	Cello.			
Carpenter and Fulton (1937)	Citrate	M.R.	V.P.	

Times the test appears in this tabulation:

Indol production	12
Methyl-red reaction	16
Voges-Proskauer reaction	15
Citrate utilization	17
Uric acid utilization	4
Cellobiose fermentation	3
Eijkman test	2
Hydrogen sulphide production	1
Gelatin liquefaction	1
Sucrose fermentation	1
Alpha-methyl-d-glucoside fermentation	1

This report is primarily concerned with coliform intermediates derived from three types of fecal material, i.e., specimens of fresh feces and certain of these specimens stored at ice box and at body temperatures. Throughout, isolation of cultures has been by direct plating. True, for each fresh specimen enrichment tubes

TABLE 2

Coliform types and their occurrence

	1	2	3	4	5	6	7	8	9
Coli									
++−−	*	+	+	+	+	+	+	+	+
−+−−			+	+	+	+	+	+	+
+−−−									
Intermediates									
+++−					+				+
++−+			+	+	+	+	+	+	+
−+−+		+	+	+	+	+	+	+	+
+−+−									
−−−−								+	
+−−+			+	+				+	
−++−			+				+		+
++++				+			+		+
+−++		+	+	+		+		+	+
−+++			+	+			+		+
Aerogenes									
−−−+			+	+			+		
−−+−			+			+	+	+	+
−−++		+	+	+	+	+	+	+	+

The formulae express in order the indol production, methyl-red reaction, Voges-Proskauer reaction and citrate utilization, i.e., the "Imvic reaction."

* 1. Koser reported five types, but did not utilize indol. Types cannot be exactly placed.

2. Minkewitsch.

3. Kline.

4. Ruchhoft et al. Recognized but four types, ++−−, −+−−, −+−+, and −−++ as fecal. Others held to be extraneous to feces or mixtures.

5. Skinner and Brudnoy.

6. Bardsley.

7. Bigger.

8. Wilson et al.

9. Parr.

of lactose broth were prepared, but they were not utilized unless direct plating failed to reveal coliform organisms. That this may happen has been shown by Parr (in press) and by Carpenter and Fulton (1937) who record occasional fecal specimens which

yield no coliform bacteria. It is felt that direct plating is the most accurate method for determining the actual flora present. The various coliform bacteria have different metabolic demands and responses. It is improbable that any enrichment method yields an entirely accurate picture of the material enriched.

The colonies chosen for study have been purified by serial inoculation into plain broth and plating on Endo's agar. Ruchhoft and co-workers (1931) rendered a valuable service in emphasizing the confusion which mixed cultures introduce into the coliform field. Due cognizance has been taken of this point.

TABLE 3

Strains repeatedly replated to check purity

TYPE	STRAINS TESTED	CHANGES
−+++	3	0
++−+	5	0
++++	6	0
+−++	6	2*
−−+−	2	0
++−−	1	0
−+−+	2	0

* At the close of the experiment the two strains of +−++ which had changed were recovered as −−++. When mixtures are plated one usually recovers the component strains. For these none was encountered.

Strains isolated which did not correspond with the types sanctioned by the Ruchhoft report were repurified and retested. In addition, as a check on the Ruchhoft point of view, which is that only four fecal coliform types exist, i.e., −+−−, ++−−, −−++, and −+−+, a number of strains were further subjected to detailed purification involving 28 serial transplants on various mediums including seven platings and pickings. In only two instances did the reactions change and in these cases we are not convinced that the cultures in question were mixtures. See table 3.

As Parr and Caldwell (1933b) and d'Herelle and Rakieten (1934) have suggested, the possibility of biochemical variation, in freshly isolated strains not yet acclimatized to laboratory me-

diums, cannot be overlooked. Among the coliform bacteria characters are from time to time lost or those in abeyance are regained. It is in part this property of the group that gives rise to the multiplicity of forms responsible for confusing results in the attempt to apply a rigid and detailed botanical type of taxonomy and makes it desirable to emphasize the "lumper" point of view rather than the "splitter" type of classification (Skinner and Brudnoy, 1932). The occasional appearance of a variant reaction may, in our opinion, be a tribute to an investigator's close touch with his strains rather than, a priori, evidence of his carelessness. Wilson and co-workers (1935) stated that most cultures positive to both the methyl-red and Voges-Proskauer reactions are mixtures. Our results (table 3 and table 4) do not confirm this view.

Purified cultures were tested according to the Imvic complex and in addition for their dissimilation of glucose, lactose, sucrose, dulcitol, inositol, salicin, cellobiose and alpha-methyl-d-glycoside, for the production of hydrogen sulphide, liquefaction of gelatin and action on milk. Gram and capsule stains and motility tests were made as indicated. In addition, a considerable number of strains were examined for their growth in boric-acid lactose broth, in sodium malonate broth, and in Jordan and Harmon's tartrate agar; for their reduction of methylene blue and for their fermentation of propylene glycol, adonitol, inulin, raffinose, mannitol, arabinose, rhamnose, maltose and xylose. Endo's agar was used for plating, Simmon's citrated agar for determination of citrate utilization and fermentation broths were prepared from Difco phenol-red broth base to which the requisite carbohydrate, glucoside or alcohol was added prior to autoclave sterilization. Levine's iron citrate medium was used for hydrogen sulphide determinations. The data on coliform intermediates from human feces are presented in table 4.

Tribute to the heterogeneity of the coliform intermediates has been paid by Werkman and Gillen (1932), Tittsler and Sandholzer (1935), Parr (1936a) and by Carpenter and Fulton (1937). In each case the reference is to intermediates of the $-+-+$ type which our data clearly show is the most important type.

In our collection of intermediates of this type we have 29 varieties. Of these, 21 are hydrogen-sulphide positive, four liquefy gelatin, one fails to ferment cellobiose, 14 fail to ferment alpha-methyl-d-glucoside and, when analyzed by their fermentations of sucrose and dulcitol into MacConkey types, six are "acidi-lactici," six are "communis," eight are "communior," and nine

TABLE 4

Fecal coliform intermediates

		FRESH FECES	ICE BOX STORAGE	37°C. STORAGE	TOTAL
Specimens		235	68	38	
Platings		235	351	183	
Strains studied		1987	1690	905	4582
Intermediates		153	473	139	765
Per cent		7.7	27.9	15.3	16.6
Types	+++−	1	0	0	1
	++−+	7	81	23	111
	−+−+	110	135	59	304
	−++−	0	6	0	6
	++++	7	122	9	138
	+−++	28	50	39	117
	−+++	0	79	9	88
Totals		153	473	139	765

Order of importance:

(1)	−+−+	−+−+	−+−+	−+−+
(2)	+−++	++++	+−++	++++
(3)	++−+ ++++	++−+	++−+	+−++
(4)		−+++	++++ −+++	++−+
(5)		+−++		−+++

are "aerogenes." This collection of $-+-+$ types includes strains from other than fecal sources, such as "infected pumps," milk, marine food, eggs, soil, water and animal pathology.

By contrast the intermediate types $-+++$, $+-++$, and $++++$ are quite homogeneous whereas the type $++-+$ though less heterogeneous than $-+-+$ is nevertheless much more so than $-+++$, $+-++$, or $++++$. None of these

intermediate types belongs to but one MacConkey group. All $-+++$, $+-++$ and $++++$ strains and all but one $++-+$ strain we have encountered ferment sucrose. The split which gives rise to more than one MacConkey group is found in the dulcitol fermentation. All these types, then, are found as both "communior" and "aerogenes" and in addition we have encountered a "communis" type $++-+$. The four intermediate types in question ferment cellobiose and alpha-methyl-d-glucoside and do not liquefy gelatin or produce hydrogen sulphide.

TABLE 5

Tabulation of tests designed to reveal coliform relationships

TYPES	M.B.-REDUCTION			PROPYLENE GLYCOL			SODIUM MALONATE			ADONITOL			BORIC ACID		
	+	−	Total	+	−	Total	+	−	Total	+	−	Total	+	−	Total
++−−	10	1	11	2	78	80	0	125	125	15	64	79	204	0	204
−+−−	0	0	0	0	13	13	0	19	19	7	4	11	5	0	5
++−+	5	6	11	4	6	10	6	4	10	8	28	36	16	0	16
−+−+	34	28	62	46	14	60	11	63	74	2	164	166	40	0	40
++++	3	11	14	11	0	11	14	2	16	36	0	36	5	0	5
+−++	6	0	6	9	0	9	12	0	12	14	0	14	1	0	1
−+++	14	2	16	8	2	10	19	2	21	43	0	43	3	0	3
−−+−	4	2	6	14	0	14	0	14	14	0	16	16	0	0	0
−−++	5	0	5	46	11	57	64	2	66	5	16	21	48	15	63
−−−+	0	0	0	2	0	2	2	0	2	1	0	1	0	0	0
Totals....	81	50	131	142	124	266	128	231	359	131	292	423	322	15	337

(Exceptions in the $++-+$ type). All $-+++$, $+-++$, and $++++$ ferment inositol and adonitol whereas the $++-+$ does not ferment inositol and most strains fail to ferment adonitol. It is interesting to note that the heterogeneous $-+-+$ type exhibits more homogeneity with respect to adonitol dissimilation than to any other test of differential value we have encountered, all but two of 166 strains failing to ferment that alcohol. The $+-++$ type is called aerogenes by a few workers. Table 5 gives the data for adonitol fermentation for some of the strains studied and includes other fragments of data which may interest students of the intermediates.

An important question is that of classification of the coliform intermediates. On this point Koser did not commit himself. Minkewitsch (1930) would recognize five species of coliform organisms as follows:

Coli sub-group:

Type I. *B. coli-communis* Escherich. Including all of its varieties and even the lactose-defective races of paracoli which are connected with typical coli by the intermediate form *B. coli-mutabile.*

Type II. *B. coli-citrovorum* Koser. Includes only the − + − + type of intermediate.

Type III. *B. coli-anaerogenes* Lembke. Acid produced, but no gas.

Aerogenes sub-group:

Type IV. *B. aerogenes* Escherich.

Type V. *B. cloacae* Jordan.

Minkewitsch's terminology requires, of course, a change in genus from *Bacillus* to *Bacterium* and we feel his intermediate species is too narrowly conceived. His concept of the inclusion of paracoli and mutating coli with typical coli seems an excellent idea. We question whether his restriction of the intermediates, the anaerogenous coli and the cloacae to a habitat in cold-blooded animals can be justified. Furthermore it is hard to justify the establishment of a coli-anaerogenes species when paracoli are included with coli. It is to be regretted that in the coliform field no application can be made of animal pathology tests and that immunological procedures have as yet found but little utilization.

Werkman and Gillen (1932) have sought to erect a genus *Citrobacter* for organisms of the coliform group producing trimethylene glycol. They regard citrate utilization, the most widely used test in dealing with intermediates, as opposed to allocation with the citrate-negative colon bacilli.

Tittsler and Sandholzer (1935) divide the coliform bacteria into the genera *Escherichia* and *Aerobacter* on the basis of the

Voges-Proskauer reaction. As they conceive them the intermediates would then be classified as *Escherichia*. Although we cannot agree with their definition of intermediates their solution of the problem of classification could be accepted, some intermediates going to *Escherichia* and some to *Aerobacter*. More fundamental, as we see it, are the objections to breaking down the genus *Bacterium*; to including citrate-positive organisms with the coli; and to the emphasis placed on the Voges-Proskauer reaction, which is based on a quantitative rather than a qualitative differentiation. Carpenter and Fulton (1937) favor the inclusion of coliform intermediates in existing genera. They hold that coli and aerogenes are distinctly different and they believe the utilization of citrate not fundamental enough to prevent classing intermediates in the same genus with coli. They suggest a classification based primarily on the Voges-Proskauer reaction, secondarily on citrate.

Our experience compels us to regard citrate utilization as a sounder basis for classification than the Voges-Proskauer reaction. Koser (1924c) made a careful study of the stability of the utilization of citrate test. He found that under a wide variety of conditions the ability to utilize citrate is not readily lost when present or acquired when not present. With this conclusion we are in substantial agreement.[1]

It is further felt that cloacal forms of aerogenes differ sufficiently from the typical aerogenes so that any consideration of separate genera should consider a split in that direction. The gelatin-liquefying, motile, unencapsulated, glycerol-negative, propylene-glycol-negative, acetyl-methyl-carbinol-negative (as regards fermentation), chinic-acid (Butcher, 1926) negative cloacae demand taxonomic contrast to the aerogenes type with these reactions reversed. Jordan (11th Edition, 1935) felt that the splitting of the genus *Bacterium* into *Escherichia* and *Aerobacter* was not warranted. To this point of view we also subscribe.

Levine (1918) emphasized the desirability for restricting spe-

[1] Rare exceptions which we have encountered are under study and will be the subject of a later report.

cies numbers through a rigid application of methods of correlation. Unless points emerge distinctly advantageous for dairy, food and soil science, for sanitation and for medicine by the erection of new genera and species their construction seems unwise. This point is particularly pertinent for bacteriology, in which field botanical and zoological methods of classification only go so far, beyond which the microbes are lifted from their natural habitat and subjected to a wide variety of tests, selection of which has to be made with the greatest of care in order to elicit data of real value and to avoid undue complexity and confusion.

Among bacteria life spans are so short that many generations pass before the observer in a few days and without the averaging, leveling influence of sexual conjugation. In a group like the coliform bacteria where contributions by animal pathology and immunological methods are largely lacking and where opportunity for bacteriophage activity is so great it seems desirable to define the organisms encountered in simple and inclusive terms. This we feel could be done with four species of the genus *Bacterium* which would include coli, intermediates, aerogenes and cloacae and their varieties.

The coliform intermediates cannot be restricted to non-human sources as Minkewitsch (1930) has suggested. In a study of the coliform flora of 446 fecal specimens Carpenter and Fulton (1937) found one in which the only organism present was an intermediate type; 14 specimens where only intermediates and aerogenes were present; 16 yielding only intermediates and coli; and 31 which contained intermediates, aerogenes and coli. Thus they found intermediates in 62 specimens of 466 studied (13.3 per cent). Parr (In press) working on the general problem of the entire coliform flora and with a wider definition of intermediates than Carpenter and Fulton used found that 21.6 per cent of 235 fecal specimens contained intermediates. Of the 100 persons submitting these 235 specimens, 31 had a flora at one time or another containing intermediates. Parr found one specimen containing only intermediates, five containing aerogenes and intermediates, 25 containing intermediates and coli, and 19 specimens contained all three sections of the coliform group.

Parr (In press) has observed that there is considerable variation in the coliform flora of the same individual from day to day. Occasionally no coliform organisms at all may be recovered. Following such a period of time, which may include several days, coliform organisms again appear. It is at such "flora crises" as these that one is apt to encounter intermediates in very large numbers. They seem to be a stage in the re-establishment or balancing of the coliform flora. At other times they are not present at all or only in small numbers. Parr feels there is evidence for believing that the human colon is not the ideal habitat for coliform intermediates. However, since they are present in from one in eight to one in four of all fecal specimens and at times in very large numbers they cannot be stripped of significance in sanitation as indicators of pollution with alvine discharges.

It is unfortunate that our knowledge of coliform intermediates does not simplify the problems of sanitary science. That such problems are somewhat clarified is true, for added evidence is given in favor of the point of view that all coliform organisms may be suspected as of human fecal origin. The occurrence of all types of coliform bacteria in the bowel is further evidence of their close and intergrading relationship. From the practical standpoint evidence points to the necessity for strict emphasis on the highest standards of personal and public hygiene as the only safeguard against the spread of enteric bacteria.

CONCLUSION

A study has been made of 765 strains of coliform intermediates derived from fresh and stored feces and of additional type strains from other sources. As a working basis for the study of coliform organisms four tests have been utilized, i.e., indol production, methyl-red reaction, Voges-Proskauer reaction, and citrate utilization. All organisms are theoretically to be considered as intermediates which occupy a position between coli and aerogenes possessing one or more characteristics of coli and one or more of aerogenes. In practice we have restricted the intermediates to those so classified by the four reactions used.

The complexity of the entire coliform group is recognized. It

is believed unwise to dignify the many differences between forms observed with taxonomic recognition. Classification for the entire group should be simple and might well be comprehended in four species of the genus *Bacterium* to include coli, intermediates, aerogenes and cloacae.

Whether coliform intermediates are essentially of fecal or non-fecal origin has not been determined. But it is certain that they do occur in stool specimens to such an extent that they must be considered for sanitary purposes as indicators of fecal pollution.

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THE EFFECTS OF SUBLETHAL DOSES OF MONOCHROMATIC ULTRAVIOLET RADIATION ON THE GROWTH PROPERTIES OF BACTERIA

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INTRODUCTION

In earlier publications (Duggar and Hollaender, 1934; Hollaender and Claus, 1936; Hollaender and Duggar, 1936) attention has been directed to certain effects of monochromatic ultraviolet radiation on bacteria and to the technique employed in this laboratory for such studies. Thus far there have been considered almost exclusively the bactericidal effects; the technique of liquid suspension cultures, including the preparation and conduct of exposure and of control cultures; and the determination of survival ratios as manifested in colony development.

The purpose of the present paper is to present certain detailed data on some of the non-lethal effects of ultraviolet irradiation and to attempt to analyze these. Since the procedures used are outlined in our previous work, only certain important modifications are mentioned here. The data given represent only a very small but typical fraction of the experimental results. Two effects were consistently apparent in all experiments in which the specified conditioning factors were maintained, and these two effects are made apparent by a comparison of growth curves of irradiated and control organisms.

MATERIALS AND METHODS

The larger part of the work was done with a strain of *Escherichia coli* furnished by Dr. P. Levine and used in this laboratory

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for several years. The main features of the results obtained were duplicated with *Serratia marcescens*. The organisms were grown on nutrient (potato-glucose) agar slants, transferred daily (except as older cultures were desired), and used after 19 to 39 hours. For any possible advantage that might be derived therefrom, the *E. coli* culture was transferred, every four weeks, to a liquid beef-broth medium, and then back again, as usual, to potato-glucose slants. For exposure the bacteria were either washed off the agar slants with a physiological salt solution,[2] shaken thoroughly, filtered several times without pressure through absorbent cotton, and used directly for exposure; or, they were washed twice, centrifuged, resuspended, shaken, and filtered again before exposure. The bacteria were exposed to the radiation in suspensions containing from 1×10^9 to 3.5×10^9 bacteria per cubic centimeter. The suspending physiological solution was non-absorbing for the wavelengths used. This high concentration of bacteria assured a high efficiency in energy utilization, and simplified the calculation of the energy required in producing the desired effects.

Monochromatic ultraviolet radiation was employed. A quartz-monochromator separated the different wavelengths, the light source being a water-cooled, high pressure, quartz, capillary, mercury vapor lamp (Daniels and Heidt, 1932). The intensity of the radiation was measured with a vacuum thermopile in connection with a high-sensitivity galvanometer, standardized against a Bureau of Standards lamp. There was an exact control of the intensity of the monochromatic radiation entering the exposure cell and of the energy absorbed by the suspension liquid, so that the effective energy absorption per bacterium could be accurately calculated. The material was exposed in a special exposure cell prepared for this work (fig. 1). Both the exposed and the control cells were located in the same temperature-controlled water bath (18°C.), and both were stirred rapidly before and during exposure. These precautions were taken to assure an actual high probability that each organism in the suspension

[2] Composition: NaCl, 6 grams; KCl, 0.4 grams; $CaCl_2$, 0.4 grams; water, 1 liter.

received equivalent quantities of radiation under identical conditions.

For the greater part of the work wavelength ca. 2650 A was used, since it proved most efficient in producing recognizable effects, and it is a band easily isolated in high purity and intensity.

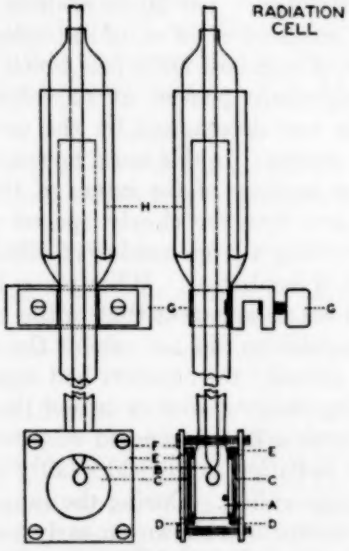

FIG. 1. EXPOSURE CELL

A, cell capacity about 6 cm.^{3}; *B*, stirrer; *C*, quartz windows; *D*, rubber gaskets; *E*, brass frame; *F*, tightening screws; *G*, for attachment to support on temperature tank; *H*, device for liquid seal.

SPECIAL CULTURAL PROCEDURES, OBSERVATIONS AND RESULTS

In the study of bactericidal effects previously reported, equivalent samples from the exposed and from the control cell were transferred, each to a unit quantity of physiological salt solution, shaken thoroughly, and then, after proper dilution, plated in potato gluose agar. It was observed that, in general, the colonies formed by bacteria which had survived irradiation appeared somewhat later than the colonies formed by the control organisms. There was a noticeable lag in development, and while no

critical study was made of that phenomenon at the time, it was at least recognized as the first prominent sublethal effect.

A second effect became apparent when it was attempted to obtain a quantitative estimate of the extent of the observed retardation by following comparatively for a few hours the growth curves of irradiated and control organisms during incubation in a liquid nutrient medium. The liquid medium adopted for such incubation series consisted of 99 cc. of salt solution with the addition of 2 to 5 cc. of standard Difco beef broth. The number of colony-forming organisms present at successive time intervals during incubation was determined by the usual agar dilution plates and colony counts. In this work, however, it was essential to obtain an exact measure of the extent of the lag phase[3] and accordingly to have data for closely spaced points along the growth curve, involving a large number of dilution plates during the first few hours of incubation. When plates from such a series were counted, it became apparent that in the exposed series the colony-forming population did not exhibit the characteristics of a typical lag but actually the numbers had apparently increased considerably during the first hour or two of the incubation, provided that the bacteria in the exposed suspension had received such quantities of radiation as to yield a fairly low survival, that is, a high percentage killing. During the same time of incubation the control culture had shown no early increase in population. Thus, there was a second effect manifest from largely-lethal intensities of ultraviolet. We shall in future refer to the first phenomenon, discussed in the preceding paragraph, as the "extended lag phase," and we shall refer to the early apparent increase (in nutrient solution) of surviving organisms which are able to form colonies on nutrient agar as the "apparent initial increase."

These two effects may be made clearer by reference to actual data. Table 1 and figures 2 and 3 illustrate a section of the results of a typical experiment, and will be explained in some de-

[3] It does not seem practicable for our purpose to distinguish between an approximately stationary phase and a subsequent slow multiplication for a time, since this may all be expressed in the idea of the lag phase of development.

tail. First, however, it should be noted that in figure 2 the actual data are plotted, whereas in figure 3 data are all recalculated for comparative effects. The bacteria (in standard salt solution) at a suspension concentration of 3.22×10^9 organisms per cubic centimeter were exposed to monochromatic ultraviolet radiation (2650 A) in the temperature bath kept at 18°C., with constant stirring, as indicated. At appropriate time intervals (actually 10, 15, 18, 21, 25, and 30 minutes) after an irradiation intensity such that each organism in the successive samples removed had received respectively 2, 4, 6, 8, 12, 20 $\times 10^{-6}$ ergs,

TABLE 1

Survival value of Run 3 = 7.7 per cent and Run 4 = 2.67 per cent

All runs diluted for incubation in 99 cc. salt solution 5 cc. bouillon

COLUMN 1	COLUMN 2	COLUMN 3	COLUMN 4	COLUMN 5	COLUMN 6	COLUMN 7	COLUMN 8
Plating	Run 3, mean of plate counts		Control for runs 3 and 4, mean of plate counts		Run 4, mean of plate counts		Incubation time
	Actual	Recalculated	Actual	Recalculated	Actual	Recalculated	
							minutes
A	230±2.64	100±1.15	322.5±6.2	100±1.92	86±2.8	100±3.26	0
B	287±7.65	123±3.3	325.5±3.84	101±1.19	98±3.57	114±4.5	40
C	334±1.48	145±0.64	326±8.56	101.2±2.66	118.7±3.53	138±4.1	75
D	385±2.31	167±1.65	387±6.1	126.1±1.89	173.3±3.65	201±4.19	117
E	405±6.57	176±2.86	657±41	239±13.4	196.3±3.65	228±4.19	160
F	653±7.93	284±3.44	1800±68	558±20.8	265.7±4.95	308±5.76	213
G	1660±23.5	722±10.2	3970±121	1240±37.7	428±9.56	498±10.2	260
H	7400±36.4	3220±15.9	1300±272	4030±34.2	1390±44.9	1620±52.2	320

accurately measured samples, of $\frac{1}{10}$ cc. each, were removed from the exposure and control cells, diluted as prearranged with physiological salt solution, and finally incubated at 32°C. in 99 cc. salt solution and 5 cc. standard Difco beef broth. A full-size series consisted of six exposures, or runs, and for such a series there were three controls, each uneven run being followed by a control. This also permitted the handling of the dilution plates in groups of three components, which were small enough for rapid work. Then by staggering the pouring of exposed and controls, rather than by pouring the one or the other successively,

a considerable possible error, arising from a longer time-interval between comparable flasks, was avoided.

We may follow, in our typical experiment as an example, a single group of "runs" (a part of the larger experiment), selecting (1) Run 3, (2) the control, and (3) Run 4—the actual data of

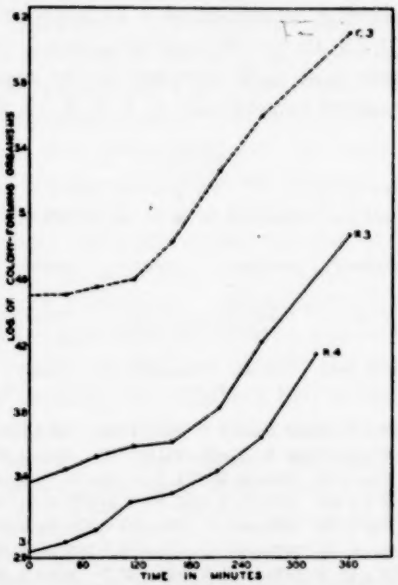

FIG. 2. *Escherichia coli*. Log of actual number of colony-forming organisms surviving in control and in irradiated (2650 A) suspensions, plated out directly after irradiation and at set time intervals. Each organism in Run 3 received 6×10^{-4} ergs, a survival value of 7.7 per cent; in Run 4 each received 8×10^{-4} ergs, a survival of 2.63 per cent. Broken line, control; continuous lines, Runs 3 and 4.

which were given in figure 2. It should be pointed out that "run" refers to any sample after an interval of irradiation and that the control in this and in certain other cases has received special attention. In keeping with the plan that the control, in selected instances, should contain at the beginning of the incubation series a number of living and of dead organisms of the order of magnitude of the irradiated suspensions, two things have been

done. In the first place, it was necessary to dilute the control appropriately with salt solution. (It was also necessary to dilute somewhat the runs from which higher survivals were expected.) Secondly, heat-killed organisms (in other cases radiation-killed) were added in requisite quantity to the control (see also page

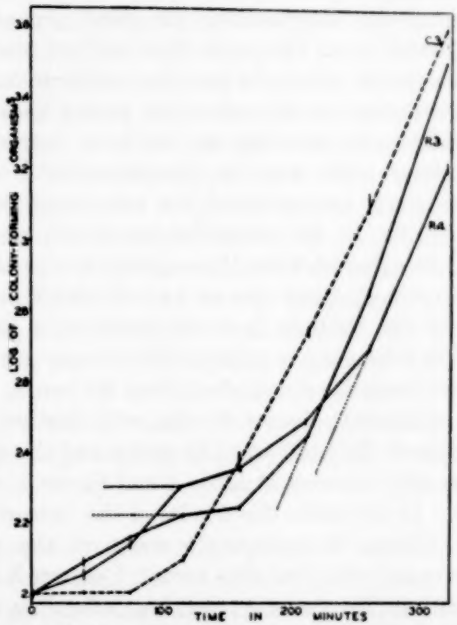

FIG. 3. *Escherichia coli.* After appropriate dilution of the suspensions (control and irradiated) for incubation and zero time platings the log of the recalculated values (see table 1), using the data of fig. 2, are plotted against time. Horizontal lines show extent of "apparent initial increase" (approximately 41 to 75 per cent) and the assurgent dotted lines indicate range of the extended lag phase.

30). In Run 3 the number inactivated, as shown by the plate counts, is 92.3 per cent of the bacteria originally able to form individual colonies, while in Run 4 the relative inactivation is 97.37 per cent. At zero time there were in Run 3 of the incubation suspension prepared as indicated 3225 organisms per cubic centimeter, of which 230 were able to form colonies when trans-

ferred to agar plates, the remainder being radiation-killed, or at least rendered unable to multiply. In Run 4 where there was a total of 3225 organisms per cubic centimeter only 86 were able to form visible colonies. The control suspension contained approximately 3600 organisms, of which the calculation indicates that 322 came from the control lot and were able to form colonies, the remainder representing the dead organisms added.

It is well also to stress the point that the first plating is made as rapidly as possible after the sample has been removed from the radiation cell and transferred to the proper flask for incubation. The subsequent platings are made at appropriate time intervals. A large series may involve 600 plates, so that both speed and accuracy are essential for consistent results. One operator is required at the monochromator and another at the platings. In all cases at least three plates are poured for each dilution, and in most cases one or two dilutions are adequate. The dilution of the bacteria is so estimated that there may be from 80 to 250 colonies per plate. The counts are made after the plates have been incubated for about 48 hours. In our experience, no additional colonies develop with further incubation.

The reliability of the results in this group and the spread of the probable error may be seen in table 1 and figure 3 (recalculated as explained). In the table the results of the successive platings are shown. Column 2 displays the mean of the actual plate count replicates and the probable error. Column 3 is, however, more important in the general overview, affording recalculated values, including the probable error. Recalculation is necessary owing to differences in population at zero time. Accordingly the recalculation is effected by considering the number of individuals in each run and each control independently as 100 at zero time. The formula for this recalculation is $m \times 100/n = p$, in which m is the uncorrected value (mean of plate counts) of any run or control at any time interval; n is the uncorrected value (mean of plate counts) at zero time; and p is the percentage value by comparison with the value at zero time. The purpose of these values is, of course, a direct comparison of growth rates. By comparison then Columns 4, 5, 6, and 7 give corresponding

values for the control (Columns 4 and 5) and for Run 4 (Columns 6 and 7). The elapsed time intervals of the platings are shown in Column 8. In figure 2 the log of the mean of the actual colony number per plate is plotted against time of incubation, whereas in figure 3 the corrected values are on the basis of 100 for each run and for the control. The value of such recalculations can be recognized by comparing figures 2 and 3.

The regularity of the normal growth curve is enhanced by the incubation of the cultures in a well-stirred water bath. Incubation in air did not yield such concordant results. The temperature of incubation was 32°C., which is not the optimum for these organisms; but the lag phase was extended at this temperature and the growth rate was such as to permit the determination of several points on the curve before the log phase was evident.

Nevertheless, it should be stressed that in order to locate such points, and additional ones, properly spaced on the growth curve, and thus bring out the characteristic growth effects, it is requisite even under the most favorable conditions to organize carefully the plating procedure and to have available an abundance of sterile media as well as vessels of every sort required. Especially essential is a large supply of perfectly delivering standardized pipettes, since no pipette may be used more than once without cleaning.

Referring again to figure 3, graphing the data discussed in the preceding paragraphs, the "apparent initial increase" and the "extended lag phase" are clearly indicated. Using Fisher's (1924) method for significant differences of small samples, the so-called "t" test, it is shown that both of these growth curve modifications referred to are statistically safe. An example of such calculations is offered in another publication (Hollaender and Claus, 1937).

Experiments with *Serratia marcescens* have yielded results confirming the two growth effects observed. In figure 4 are displayed growth curves of this species representing a series of three exposures and two controls. Both the apparent initial increase in the number of colony-forming organisms and the extended lag phase are obvious by comparison of the behavior of the exposed

and the control cultures. In this case the exposed cultures in Runs 1, 2, and 3 represent respectively survival values of 41, 11, and 2.6 per cent. Note the apparent initial increase at the lower survival value. No other species of bacteria have been tested, since the work with *E. coli* alone has required a considerable number of experiments necessitating handling of many thousand plates; and it has seemed more desirable for the present to study

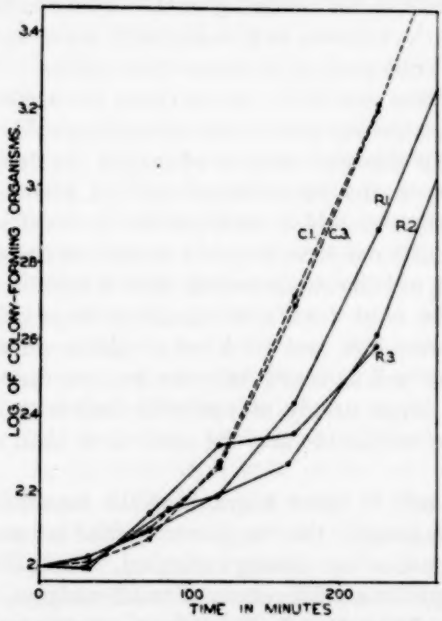

FIG. 4. *Serratia marcescens*. Growth curves of control and irradiated cultures recalculated as in fig. 3 from the original data. Complete indications in text.

this organism intensively than to obtain limited data with an assortment of organisms.

INFLUENCES AFFECTING THE APPEARANCE OF THE GROWTH PHENOMENA OBSERVED

Selection for the experimental work of cultures grown from 15 to 39 hours at 32°C. has been made on the basis of experience, since the age of the culture affects the response to radiation. In

figure 5 are given, as examples, growth curves for a 15-hour and a 48-hour culture. Cultures younger than 15 hours gave a lag phase too short to make possible the effective determination of an adequate number of points in this part of the curve.

Washing the bacteria has no direct effect on the appearance of the special phenomena here considered, although somewhat less energy is required with washed organisms to produce these ef-

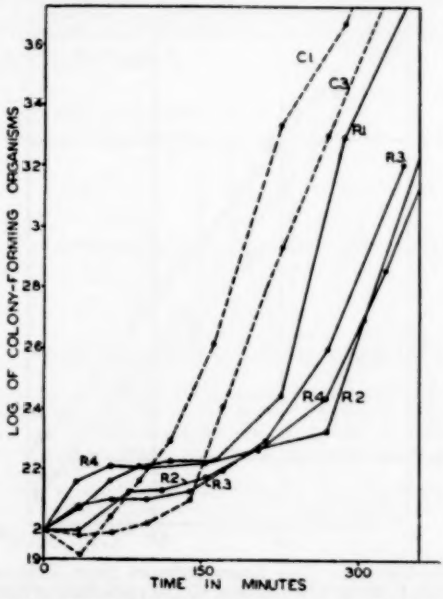

FIG. 5. *Escherichia coli*. Growth curves of control and of irradiated cultures, recalculated as in fig. 3 from the original data. *R.1* and *R.2* were exposed to different energy values (*R1* < *R2*); these and *C1* (control) were from a 15-hour culture, while *R.3*, *R.4*, and *C.3* were from a 48-hour culture, but treated in a manner identical with the first group.

fects, and this is in accord with earlier findings that the efficiency of bactericidal action is increased slightly after washing. That no detectable effect is produced on the suspending liquid under the conditions applying is of much importance. Special tests were made by irradiating the salt solution and observing comparatively the growth properties of bacteria in it and in a control,

after the addition of sufficient beef broth to afford growth. This refers only to wavelengths and energies used in this investigation.

In order to display the relation of the effects here discussed to bactericidal action, figure 6 is introduced, showing the results in the form of a typical killing curve at 2650 A. The logarithm of the survival value is plotted against the energy necessary to kill one organism. This graph is divided into four sections, *A*,

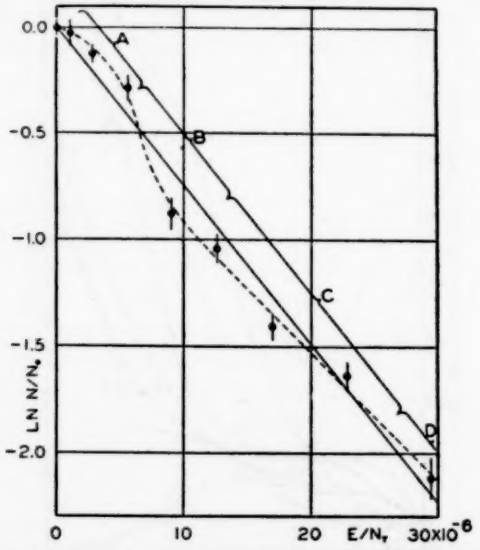

FIG. 6. *Escherichia coli*. This figure redrawn from Hollaender and Claus (1936), and extended, shows the relation of the apparent initial increase and of the extended lag to bactericidal action. While the extended lag appears in sections *B*, *C*, and *D*, the apparent initial increase is in section *C* alone.

B, *C*, and *D*. The extended lag phase will appear in sections *B*, *C*, and *D*, the apparent initial increase in section *C* only.

Quantitative response is obtained, depending on the energy applied. As a final test of the types of quantitative response several complete experiments were made. In these, at least six exposures and three controls were included. During the irradiation, successive samples were removed to afford increasing energies, and growth curves for each energy level were followed.

It was then found that the apparent initial increase is governed very closely by the percentage killing (and no doubt by the general procedure to a lesser degree). A concrete case will illustrate this point. If 1,000,000 are irradiated and 999,900 are killed the surviving 100 organisms may not show the initial effect, certainly not in pronounced form; but if 980,000 are killed, the surviving 20,000 will show probably the highest possible increase. On the other hand, when 999,900 are killed the surviving 100 will show the most pronounced extended lag. Two figures (7a and 7b) illustrate the point discussed.

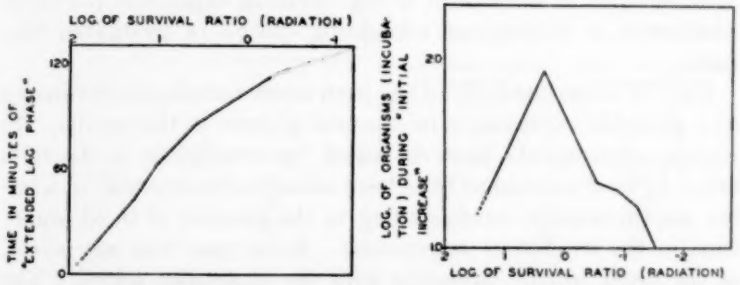

FIG. 7a. *Escherichia coli*. This graph shows the time of the extension of the lag phase plotted against the log of the survival ratio; it illustrates the time elapsed after the controls have started to divide before the irradiated bacteria go into their *normal* growth phase, and the dependence of the extension on the amount of radiation used.

FIG. 7b. *Escherichia coli*. This curve shows the magnitude of the initial increase, that is, the increase (log) of colony-forming organisms in the irradiated suspensions plotted against the log of the survival ratios.

The maximum display of the apparent initial increase occurs at around 95 per cent killing (actually 94 to 98 per cent), and decreases with lower or with higher survival ratios, none of this effect being observable in sections *A*, *B*, and *D* of the inactivation curve (fig. 6). Its occurrence within so limited a set of survival values has no doubt obscured this phenomenon. On the other hand, the extended lag phase will appear over a relatively wide range of values, although more pronounced when survival is down toward one percent. At very low survival values the extended lag phase is, in fact, recognizable even in the delayed appearance of the colonies on the agar plate.

Perhaps it is necessary to defend or explain that part of our procedure in which dead (irradiation-killed or heat-killed) bacteria are added to the control groups attempting to simulate exposure conditions, with the possibility of establishing some causative relations of the irradiation to the observed effects, especially in the case of the apparent initial increase in the number of colony-forming organisms. There was the possibility (1) that materials diffusing out of the radiation-killed (as well as out of radiation-surviving) bacteria might produce a "stimulating" effect, or (2) that differential effects might be produced as a result of differences in number of living, dividing organisms per cubic centimeter in suspensions containing control or irradiated bacteria.

Each of these possibilities has been investigated and eliminated as a plausible explanation or a source of error in the results. In various experiments bacteria killed by irradiation or bacteria killed by heat treatment have been added to the controls in number approximately corresponding to the number of dead organisms in the irradiated suspensions. In no case was any effect, of the order sought, apparent with the quantities added. The energy used for killing the bacteria by irradiation was no more than was necessary to attain 100 per cent efficiency, since it is to be assumed that at very high energies changes might be induced in the dead bacteria which might have either a beneficial or an injurious effect (Blank and Arnold, 1935).

The concentration of the nutrient beef broth seemed not to have been a very important factor in the appearance of the effects discussed. A low concentration nutrient medium was selected for convenience. The pH of the medium had, as far as tested, no differential effect on control and irradiated suspensions. The reality of the effects described can be seen both from the experiments in which each step has been done in quadruplicate (Hollaender and Claus, 1937, p. 80) and also from the tests previously made on the delay of the two new phases as induced by keeping the irradiated and the control suspensions, after irradiation, in a bath at 0°C., for a certain time interval (l. c., p. 87). The final yield, after extended time, was apparently the same in

the flasks containing the control as in that containing the exposed organisms. Apparently control and exposed organisms grow until the nutrient material is used up, or until toxic substances formed make further growth impossible. Nevertheless, additional work on this point is very desirable.

It is not infrequently stated that in making a transfer from an old culture of bacteria to a fresh medium the lag or resting phase may be shortened by using as inoculum a relatively large number of organisms per cubic centimeter rather than a small number (cf. Buchanan and Fulmer, 1928, vol. 1). While relative quan-

TABLE 2

MEAN NUMBER OF COLONIES EXPOSED	MEAN NUMBER OF COLONIES CONTROL	EXPOSED PLUS CONTROL CALCULATED	EXPOSED PLUS CONTROL EXPERIMENTAL
124	86.5	210.5	236
139	81.7	220.7	238
202	94	296	258
230	94	324	272
250	147	397	402
300	190	490	506
93	80.2	173.2	156.3
88	85	173	170
143	90	233	196
147	93	240	210
192	152	344	335
520	482	1002	802

tity of inoculum could hardly play a rôle in the experiments here described, the matter has received attention and precautions were taken. The population of the inoculum was so adjusted that the number of colony-forming organisms in each incubation flask was comparable at the beginning; thus, if the indications were that 80 per cent of the organisms had been killed by irradiation, greater dilution was made than if the inactivation had affected 98 per cent.

Table 2 exhibits the results of two experiments in which, immediately following the irradiation interval, two separate incubation series were set up, and parallel with these a mixed suspension

of irradiated and control organisms was arranged. With the development from zero time the number of colony-forming organisms was determined in the usual manner, that is, by dilution plates made at set intervals, and the counts were also made as usual. In column 1 the means of the plate counts derived from the irradiated suspension are displayed and in column 2 corresponding figures for the unirradiated control. The data in column 3 have been obtained by adding columns 1 and 2. These last figures represent "calculated" values for the mixed solution in the circumstance that irradiated organisms mixed with control organisms do not recognizably alter the growth rate of the control. In column 4 are given the means of the actual plate counts furnished by the mixed suspension prepared as previously indicated. The agreement between the corresponding figures in columns 3 and 4 is satisfactory, suggesting the absence of any effect of irradiated upon unirradiated organisms under the conditions of these experiments.

DISCUSSION

It should perhaps be made clear that the energies employed in this work are far beyond the order of magnitude of those considered to be involved in the alleged "mitogenetic radiation." In the study here reported, the energies are in the neighborhood of 2000 ergs per cm.^{2} per sec., whereas the energy claimed for mitogenetic radiation is 10^{-9} to 10^{-11} ergs per cm.^{2} per sec., so that it seems unnecessary to take this point further into consideration.

The effects reported here are on the organisms; and, as previously indicated, they are not the result of changes in the suspending liquid.

Since growth properties exhibited by the organisms surviving irradiation at the intensities here considered, in comparison with the control organisms, constitute the chief interest in this work, it seems well to emphasize certain conditions of the culture technique. After irradiation, suitable dilutions of the irradiated and control suspensions are made in a beef broth medium. Zero-time dilution cultures are made on agar plates. Samples from

the (1) irradiated and (2) control are then incubated as parallel series in the broth medium and platings are made at various intervals. All conditions are similar for the two series.

It may be well to consider separately the provisional interpretations of the "extended lag" and of the "apparent initial increase." The most reasonable explanation of the "extended lag" phase may be found perhaps in the assumption that the bacteria have been somewhat injured during the process of irradiation, but not sufficiently to impair permanently their ability to divide. Whether the injury is produced by direct or indirect effects cannot be decided at the present stage of this investigation. Any explanation which is adequate must take into consideration the fact that the "extended lag" is not eliminated by any preceding "early apparent increase." It is well to recall also that the addition of irradiated to control organisms affords no evidence that the control organisms in the mixture are affected by materials transferred with the irradiated bacteria. It is also possible that the bacterial cell contains enzymes or moderately photosensitive compounds which respond to such quantities of energy as are specified in this work, despite the fact that the destructive effects of ultraviolet radiation on enzymes ordinarily require considerably more energy than is necessary to induce the "extended lag." That this lag extension has not earlier received more attention is probably a result of the methods of investigation employed, these methods making it difficult, if not impossible, to recognize effects other than those which are purely lethal (cf. footnote p. 20). From a purely biological viewpoint, the occurrence of a recovery time after injury is reasonable.

There seems to be, unfortunately, no equally reasonable explanation of the early apparent increase. In the several paragraphs following, an attempt is made to analyze certain of the possibilities.

Clumping, as a result of irradiation, and de-clumping, after transfer to broth, came up as a possibility; but the idea is not supported by any evidence we have been able to obtain from the results of vigorous shaking or from microscopic study.

The problem of the microscopic study of the phenomenon is

frankly a difficult one. Evidence from microscopic study would be more easily obtained with other organisms. The difficulty of establishing such a point by a microscopic count of cells may be made clear from the following. If, in an irradiated suspension of unit quantity, there is a total of 1000 individuals, 100 of which increase during the early incubation period to 150 or 200, the original 1000 becomes 1050–1100, and this represents a change in total cell number of from 5 to 10 per cent. Such an increase cannot be established accurately by means of microscopical cell counts. Furthermore, microscopical counts are less accurate in very low concentrations, whereas the initial increase comes out more clearly in high dilution of the incubated suspension. We shall postpone further discussion on this basis until our studies on yeast, to be reported later, are available.

It appears that in the suspension of bacteria employed in the incubation series, there are about 5 to 10 per cent of double cells. These are present in spite of vigorous shaking and filtering, but the segregation of these would not account for the observed increase of 30 to 40 per cent or more.

The early apparent increase in the number of colony-forming organisms might seem to arise as a result of a prompt recovery of a certain percentage of cells, followed later by the characteristic lag which leads into division at the normal rate. This again would ignore the fact that "recovery" should then be manifest when the zero-time plating is made. There is the further fact that the extended lag phase is evident with 70 per cent survival; whereas, the apparent initial increase appears around 20 per cent survival, and is more pronounced with 8 per cent or less. It would appear that simple recovery should be most pronounced at the high survival values, since there the cells would be least damaged.

In almost every contribution on the bactericidal effect of ultraviolet radiation there is some mention of the possibility of growth "stimulation" by means of these wavelengths (see the review by Duggar, 1936). In most of these papers the evidence is interpreted as negative, but there are exceptions, such as those of

Browning and Russ (1917). However, we agree with Bachem and Dushkin (1933) in the interpretation that the so-called stimulation in this case was merely the growth of larger colonies (with more space and available nutrients) in areas of exposed plates where survival was not high. Bovie (1916) suggested (without explanation) that partial decomposition of the protein "may stimulate the cell." Coblentz and Fulton (1924) report as follows: "There is some evidence that organisms are stimulated in growth when exposed to low intensity ultraviolet radiation." No experimental data were included. Certain morphological modifications resulting from irradiation have been observed by Gates (1933). In this short note, without special data, referring to "degenerative" changes in organisms strongly irradiated but not killed, he lists "gradual degeneration," division into "large or small units, which then degenerate," and "daughter cells may pinch off at one end and multiply rapidly to colony formation."

The data presented indicate that a significantly small surviving population from a given light intensity exhibit on incubation an "apparent initial increase." Is this "increase" the result of early cell multiplication? If the individuals divide promptly on incubation, why do they not continue, in a nutrient medium, to divide rather than pass into a typical lag? If cell division is assumed, what is a plausible suggestion regarding the mechanism?

From the observations recorded in this paper little or no support is given the possibility that the initial increase is either a recovery phase or a loss of clumping property; rather the evidence seems to point to the hypothesis—not yet verified by all possible means of attack—that the "apparent initial increase" described is actually a multiplication of cells. Any hypothesis regarding the mechanism of the extended lag phase should take into consideration the probability that over and above the intracellular products that may determine the normal lag there is doubtless a further addition of inhibiting substances as a result of irradiation. All these, however, must play a secondary role as long as the division-promoting substance is present in sufficient concentration.

This work was conducted with the support of the Committee on Radiation, Division of Biology and Agriculture, National Research Council.

SUMMARY

1. Two effects of sublethal doses of ultraviolet radiation were observed; (*a*) an apparent initial increase in number of colony-forming organisms, and (*b*) an extension of the lag phase.

2. Methods and procedures were developed for the irradiation process and the recognition of the sublethal effects.

3. These effects have been illustrated by typical experiments.

4. The influence of a number of factors on the appearance of the phenomena have been followed, e.g., treatment of the organisms before, during, and after irradiation, giving special attention to the reproducibility of the effects.

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THE CLASSIFICATION OF ACID-FAST BACTERIA. II

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In a previously reported attempt (Gordon, 1937) to find standards for the classification of the rapidly-growing, non-pathogenic mycobacteria, we presented the results of a cultural study of a collection of 252 strains from soil, plants, human and animal tissues and secretions. Eighty per cent of the strains were readily separated into three groups of closely-related cultures. The remaining 20 per cent of the strains, which differed from the above and in most cases from each other, were arbitrarily distributed among the three major groups as sub-groups. Since 84 per cent of the cultures of the collection had been isolated from soil by means of Söhngen's technique, there arose the question of whether cultures from sources other than soil would fall into the same groups and whether the standards previously set would be adequate. It seemed possible that some of the unrelated, single strains of our soil collection might be represented in large numbers in an assemblage of cultures from other sources and isolated by other methods.

Seventy-nine additional strains of saprophytic acid-fast bacteria have been studied by the same methods described in our previous report. The numbers and sources of the cultures are recorded in table 1. The fish strains were isolated on glycerinated egg medium; the soil and water cultures were obtained by Söhngen's technique (1913).

EXPERIMENTAL

Two of the three major groups, first devised by Thomson (1932) for the classification of the saprophytic acid-fasts and

employed in our previous study, were represented in the more recent collection. None of the cultures were of the *Mycobacterium phlei*, or Group III, type. Seventeen strains, fifteen of which are of the *Mycobacterium smegmatis* type, were placed in Group I. The remaining cultures were assigned to the more heterogenous Group II.

The cultures have been grouped according to a plan which differs slightly from our first arrangement. In Group I, the two sub-groups, which previously had been separated on the basis of arabinose utilization, were separated on the basis of utilization

TABLE 1

NUMBER	CULTURE	SOURCE
11	Human and rat leprosy	American type culture collection
1	Bovine tissue	E. G. Hastings
1	Sputum	L. V. Gardner
1	Saprophytic strain*	Dairy department, Cornell University
10	Spontaneous tuberculosis of killifish (*Platypoecilus maculatus*)	J. A. Baker
39	Water in killifish aquaria	J. A. Baker
16	Local soil	R. E. Gordon

* This culture came to us labelled "The original Koch strain of human tuberculosis." Acting upon the recommendation of Hastings and McCarter (1932), who inquired into the history of and studied several so-called saprophytic human and bovine tubercle bacilli, we have not used this name.

of arabinose, sorbitol, galactose, trehalose, mannitol, and fructose. In Group II, a new sub-group, IIc, was created for the disposal of those cultures which did not utilize any of the carbohydrates employed. The altered plan and the grouping of the new cultures are as follows:

Group I

Fails to survive 60°C. for one hour.
Grows at 47°C.

a. Utilizes arabinose, sorbitol, galactose, trehalose, mannitol, and fructose. Fifteen strains similar in appearance and cultural reactions.

b. Unable to utilize all the above carbohydrates. Two strains differing in appearance and cultural reactions from the above cultures and from each other.

Group II

Fails to survive 60°C. for one hour.
Does not grow at 47°C.

a. Unable to utilize sorbitol.
   1. Unable to utilize arabinose. Forty strains similar in appearance and cultural reactions. Two dissimilar strains.
   2. Utilizes arabinose. Two similar strains.
b. Utilizes sorbitol. Eight dissimilar strains.
c. Unable to utilize any of the carbohydrates tested. Ten similar strains.

Group Ia. The strains assembled in this group produced a characteristic growth and color change. The growth was rapid, spreading, and wrinkled. At first, the growth was a dull, creamy white, but after four to six weeks at room temperature the cultures became a deep yellow or orange. All of the test carbohydrates, except sucrose, were utilized. Of the fifteen cultures placed in this group, ten were soil isolations; one strain had been isolated from sputum of a tuberculosis patient; another strain was the so-called saprophytic tubercle culture; and the remaining three strains bore the name, *Mycobacterium leprae.* Two of the latter had been isolated from leprous rats; the third was the culture of Levy-Kedrowski.

Group Ib. This group was created for the disposal of cultures which grew at 47°C., but were not of the *Mycobacterium smegmatis* type. Four dissimilar cultures, two leprae strains and two soil isolations, had been assigned to this group in the previous study. Two more cultures, the leprae strain of Lombardo-Pellagrine and the isolation from bovine tissue, were added from the new collection. The resulting six cultures were dissimilar in appearance and biochemical reactions.

Group IIa$_1$. The predominating culture type of this group produced a creamy white growth which did not change color with aging. It was either rough or smooth in type; it utilized fructose and, in most instances, trehalose and mannitol. Forty cultures,

one a soil isolation and thirty-nine water strains, were assigned to this group.

Another type placed in this group showed the same biochemical characteristics as the predominating type, but produced a yellow pigment. Our first collection contained ten of these yellow pigmented strains, and our second collection had one, a soil isolation.

The remaining dissimilar strain assigned to this group, *Mycobacterium leprae* of Brinkerhoff, is a smooth, pale yellow culture which utilized mannitol, trehalose, fructose, and sucrose. It was similar in appearance and biochemical reactions to our Duval leprae strain previously included in this group.

Group IIa$_2$. The two smooth, salmon-pink, soil cultures placed in this group utilized arabinose, galactose, trehalose, mannitol, and fructose. These cultures resembled none of the seven dissimilar cultures previously assigned to this group.

Group IIb. The eight cultures assembled in the group represent six different types. A smooth, yellow soil isolation and the leprae strain of Needham appeared to be identical in appearance and cultural reactions. They utilized all carbohydrates tested and resembled two soil isolations previously described.

A rough, creamy white, soil culture utilized sorbitol, mannitol, trehalose, and fructose and was similar to three soil isolations previously included in this group.

The remaining five leprae strains were nearly identical in biochemical characteristics, but varied greatly in appearance. The smooth, salmon-pink, leprae culture of Levy-Chrome was apparently identical with the leprae strains of Brinkerhoff and Clegg previously studied. The red-pigmented culture of Rost-Williams resembled a soil isolation of our former collection. The rat leprosy strain of McCoy resembled the leprae strain of Elly previously included in this group. The two pale-yellow, pigmented cultures of Currie and Barry seemed closely related, if not identical, with each other.

Group IIc. The ten yellow pigmented cultures isolated from spontaneous tuberculosis of killifish (*Platypoecilus maculatus*) utilized none of the carbohydrates employed. They would not grow at 37°C., nor on Söhngen's medium. They were not identi-

cal with any culture in our former collection, although seven of our first cultures, including *Mycobacterium cheloni* and *Mycobacterium schlangen*, did not utilize any of the seven carbohydrates. These seven cultures were previously placed in Group IIa_1. It seemed advisable to create a new subdivision

TABLE 2

Cultural characteristics and grouping of our collection of saprophytic, acid-fast cultures

GROUP	NUMBER OF CULTURES	SURVIVAL AT 60°C. FOR 60 MINUTES	GROWTH AT 47°C.	GROWTH ON MERRILL'S MEDIUM WITH VARIOUS CARBON SOURCES							GROWTH ON DORSET'S SYNTHETIC MEDIUM, NO GLYCEROL	ALKALI PRODUCTION IN LITMUS MILK	NITRATE REDUCTION
				Sorbitol	Arabinose	Galactose	Trehalose	Mannitol	Fructose	Sucrose			
Ia	120	−	+	+	+	+	+	+	+	−	−	+	116 + 4 −
Ib	6*	−	+	±	±	±	+	±	+	±	−	+	±
IIa_1	106	−	−	−	−	−	±	±	+	−	−	+	±
	11	−	−	−	−	−	+	+	+	−	−	+	+
	6*	−	−	−	−	−	±	±	+	±	−	±	±
IIa_2	9*	−	−	−	+	±	±	±	+	±	−	±	±
IIb	16*	−	−	+	−	−	+	+	+	±	−	+	±
	10*	−	−	+	±	±	±	±	+	±	−	+	±
IIc	10	−	−	−	−	−	−	−	−	−	−	sl	±
	7*	−	−	−	−	−	−	−	−	−	−	±	±
IIIa	29	+	+	+	+	+	+	+	+	−	±	+	±
IIIb	1	+	+	sl	−	sl	+	+	+	−	−	sl	+

* Dissimilar cultures.

of Group II and to assign to it these seventeen comparatively inactive cultures.

A compilation of the data on all the cultures we have studied is presented in tables 2 and 3. The new plan of culture-grouping affected the arrangement of the cultures first studied only slightly, the one change being the assignment of seven strains previously

TABLE 3

Source and grouping of our collection of saprophytic, acid-fast cultures

GROUP	NUMBER OF CULTURES	NAME OF CULTURES	SOURCE	ISOLATED BY
Ia	1	*M. smegmatis*	Smegma	Alvarez and Tavel
	2	*M. graminis*	Plant dust	Moeller
	2	*M. stercusis*	Cow manure	Moeller
	1	*M. berolinense*	Butter	Rabinowitch
	1	*M. paratuberculosis*		Binot
	1	*M. pseudoperlsucht*		
	1	Grassberger's bacillus	Butter	Grassberger
	1	*B. aus Nasenschleim*	Nasal exudate	Karlinski
	1	Traum's bacillus	Bovine lymph gland	Traum
	1	*M. leprae*	Human leprosy	Levy-Kedrowski (A.T.C.C.)
	2	*M. leprae*	Rat leprosy	Am. Type Cul. Collection
	1	Saprophytic Koch strain		
	1	Unnamed	Sputum	Gardner
	104	Unnamed	Soil	Cornell University
Ib	1	*M. leprae*	Human leprosy	Clegg
	1	Kat #352	Human leprosy	Carpenter
	1	*M. leprae*		Lombardo-Pellagrine (A.T.C.C.)
	1	Unnamed	Bovine tissue	Hastings
	2	Unnamed	Soil	Cornell University
IIa_1	1	*M. ranae*	Frog's liver	Kuster
	1	*M. thamnopheus*	Garter snake	Aronson
	1	*M. leprae*	Human leprosy	Duval
	1	*M. leprae*	Human leprosy	Brinkerhoff (A.T.C.C.)
	39	Unnamed	Water	Cornell University
	80	Unnamed	Soil	Cornell University
IIa_2	1	*M. marinum*	Fish	Aronson
	1	Lymph gland acid-fast	Bovine lymph gland	Hastings
	7	Unnamed	Soil	Cornell University
IIb	1	*M. leprae*		Lister Institute #518
	1	*M. leprae*		Elly
	1	*M. leprae*	Human leprosy	Brinkerhoff
	1	*M. leprae*	Human leprosy	Clegg
	1	*M. leprae*	Rat leprosy	McCoy (A.T.C.C.)
	1	*M. leprae*	Human leprosy	Currie (A.T.C.C.)
	1	*M. leprae*		Barry (A.T.C.C.)
	1	*M. leprae*	Human leprosy	Rost-Williams (A.T.C.C.)
	1	*M. leprae*	Human leprosy	Levy-Chrome (A.T.C.C.)
	1	*M. leprae*		Needham
	1	Wong strain	Human leprosy	Carpenter
	1	Unnamed	Skin lesion of calf	Daines
	14	Unnamed	Soil	Cornell University
IIc	1	*M. chelonei*	Turtle's lung	Friedmann
	1	*M. schlangen*	Snake	Sibley
	1	Bayne-Jones' acid-fast	Human lung	Bayne-Jones
	1	Plum's bacillus	Milk	Plum
	1	Guernsey heifer acid-fast	Bovine tissue	Hastings
	2	Unnamed	Soil	Cornell University
	10	Unnamed	Fish	Cornell University
IIIa	2	*M. phlei*	Timothy grass	Moeller
	1	Unnamed	Hen's spleen	Cornell University
	2	Unnamed	Bovine lymph gland	Cornell University
	1	Zeissig's bacillus		Cornell University
	22	Unnamed	Soil	Cornell University
	1	Unnamed	Bovine skin lesion	Cornell University
IIIb	1	Hog's skin bacillus	Hog's skin	Buckley

placed in Group IIa_1 to the new sub-group IIc. Of the total 331 cultures, 276 strains fell into four groups of closely related cultures, while the remaining 55 strains, or seventeen per cent of the total number, were temporarily distributed among the various sub-groups.

TABLE 4

Grouping and cultural characteristics of leprae strains

NAME OF ORIGIN	SURVIVAL AT 60°C., FOR 60 MINUTES	GROWTH AT 47°C.	GROWTH ON MERRILL'S MEDIUM WITH VARIOUS CARBON SOURCES							GROWTH ON DORSET'S SYNTHETIC MEDIUM ON GLYCEROL	REDUCTION OF NITRATES	ALKALI IN LITMUS MILK	GROWTH ON SÖRGEN'S MEDIUM
			Sorbitol	Arabinose	Galactose	Trehalose	Mannitol	Fructose	Sucrose				
Group Ia:													
M. leprae, rat 2, A.T.C.C.	−	+	+	+	+	+	+	+	−	−	+	+	+
M. leprae, rat 1, A.T.C.C.	−	+	+	+	+	+	+	+	−	−	+	+	+
M. leprae, Levy-Kedrowski	−	+	+	+	+	+	+	+	−	−	+	+	+
Group Ib:													
M. leprae, Clegg I	−	+	+	−	+	sl	+	+	−	−	+	sl	+
M leprae, Lombardo-Pellagrine	−	+	+	−	−	+	+	+	−	−	+	+	+
Human leprosy, Carpenter	−	+	+	−	−	+	+	+	−	−	+	+	+
Group IIa_1:													
M. leprae, Duval	−	−	−	−	−	+	+	+	+	−	−	sl	+
M. leprae, Brinkerhoff	−	−	−	−	−	+	+	+	+		−	+	+
Group IIb:													
M. leprae	−	−	+	−	−	+	+	+	−	−	+	sl	+
M. leprae, Elly	−	−	+	−	−	+	+	+	−	−	+	sl	+
M. leprae rat, McCoy	−	−	+	−	−	+	+	+	−	−	+	+	−
M. leprae, Brinkerhoff II	−	−	+	−	−	sl	+	+	−	−	+	sl	+
M. leprae, Clegg II	−	−	+	−	−	+	+	+	−	−	+	sl	+
M. leprae, Levy-Chrome	−	−	+	−	−	+	sl	+	−	−	+	sl	+
M. leprae, Currie	−	−	+	−	−	+	sl	+	−	−	+	sl	+
M. leprae, Barry	−	−	+	−	−	+	+	+	sl	−	−	sl	+
M. leprae, Rost-Williams	−	−	+	−	−	+	+	+	+	−	+	+	+
M. leprae, Needham	−	−	+	+	+	+	+	+	+	−	−	+	−
Human leprosy, Carpenter	−	−	sl	−	−	−	−	+	−	−	+	+	−

Our entire collection now contains nineteen strains named *Mycobacterium leprae*. These cultures show considerable variation both in appearance and cultural characteristics (table 4). As previously stated, some of them (five) could not be distinguished from certain soil isolations. Ten strains (two pale-yellow pigmented, four deep yellow, and four salmon-pink) of the

nineteen utilized sorbitol, but not arabinose or galactose, and did not grow at 47°C. Only seven of our soil isolations showed this combination of reactions, but they did not resemble any of the leprae strains in appearance. Attempts to separate the leprae cultures more definitely from the soil strains have been unsuccessful.

SUMMARY

Seventy-nine acid-fast cultures from cases of human and rat leprosy, bovine tissue, sputum, fish, water and soil were separated into two major groups according to previously set standards. Sixty-five cultures (eighty-two per cent) were readily assigned to closely related groups. The remaining fourteen strains were arbitrarily distributed among four heterogeneous sub-groups.

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A STUDY OF GELATIN DIGESTION BY BACILLUS SUBTILIS

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The following research was started through the observation that the rate of gelatin decomposition by *Bacillus subtilis*, measured by formol titration, continued to increase for at least 5 days after the cells had reached their maximal number. This behavior which differs fundamentally from that of sugar fermentations was finally explained by the fact that decomposition of gelatin is brought about by an extracellular enzyme while sugar fermentations are due to endo-enzymes.

The rate of any fermentation is proportional to the number of enzyme molecules. In fermentations brought about by endo-enzymes, such as lactic, alcoholic and colon fermentations, the enzyme concentration is proportional to the number of cells, for it seems reasonable to assume that the average enzyme content per cell is fairly constant. Thus, when the maximal number of cells has been reached, the enzyme concentration can increase no more, and the maximal rate of fermentation is reached at this point. One of the authors (Rahn, 1910) has worded this somewhat differently: "The point of inflection (of the curve of products plotted against time) indicates the moment when the maximum number of bacteria is present." Accumulation of products may retard the rate even before the maximal number of cells has been reached, as e.g., in the lactic fermentation, figure 1.

This rule does not apply to extracellular enzymes. They are comparable to secreted metabolic products of the cell. They are produced either as long as the cell lives, or until old age retards all metabolism, or until the enzyme concentration of the medium

prevents further enzyme formation. Such enzymes are very probably secreted even after multiplication of cells has ceased. While the cell number begins to decline, the enzyme concentration still increases, and the rate of decomposition may become more rapid for some time.

METHOD

Three hundred and fifty cubic centimeters of a 5 per cent gelatin solution, inoculated with 5 cc. of a broth culture of *Bacillus*

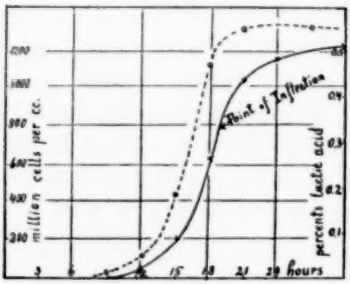

FIG. 1

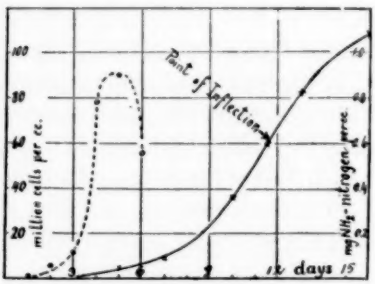

FIG. 2

FIG. 1. ACID FORMATION BY STREPTOCOCCUS LACTIS
Full line = per cents of lactic acid; dotted line = cells per cubic centimeter.

FIG. 2. NH_3 FORMATION BY BACILLUS SUBTILIS
Full line = milligrams NH_3-nitrogen per cubic centimeter; dotted line = cells per cubic centimeter.

subtilis, was incubated at 37°. Plate counts were made daily until the cell numbers began to decrease, and formol titrations were made frequently until constancy.

The formol titration was carried out by diluting 10 cc. of the culture with 10 cc. of water. This mixture was titrated with NaOH and phenolphthalein to a deep-red endpoint. Ten cubic centimeters of formalin adjusted to the same endpoint was added, and after the reaction had taken place for one minute, the sample was titrated again to the same endpoint. The difference in titration values gave the milligrams of amino and ammonia nitrogen per cubic centimeter of culture.

RESULTS

Table 1 and figure 2 show a typical result. The cells reach their maximum on the 5th day while the amount of gelatin de-

composed per day increases until after the 10th day, which is quite evident from the upward trend of the products curve. The calculation of products per cell per hour which, in case of lactic or alcoholic fermentation, gives at first a constant rate and later rapidly decreasing values, shows here an increase until the 12th day.

The assumption made above that the enzyme is extracellular needs proof for it may be that the partially decomposed gelatin

TABLE 1

Amounts of amino and ammonia nitrogen produced from gelatin

TIME	PLATE COUNT	$NH_2 + NH_3$ NITROGEN PER CUBIC CENTIMETER OF CULTURE		
		Total	Daily increase	Per cell per hour
days	*cells per cc.*	*mgm.*	*mgm.*	*10^{-10} mgm.*
0	24,000	0.304	0	
1	520,000			
2	6,300,000	0.311	0.0035	0.081
3	11,000,000			
4	78,000,000			
5	90,000,000	0.352	0.0136	0.18
6	56,000,000			
7		0.395	0.0215	0.13
10		0.661	0.0886	0.53
13		1.130	0.1563	0.93+
16		1.390	0.0867	0.52
20		1.570	0.0450	0.26
26		1.680	0.0183	
30		1.700	0.0036	
32		1.700	0	

diffuses into the cells, and the actual formation of NH_2 groups is an intracellular process after all. Probably, part is actually intracellular, but this fraction cannot be large as the following experiment shows.

One-half of a 9-day-old culture was filtered through a Berkefeld filter, and the formol titration showed that gelatin decomposition of the filtrate continued in a straight line at the rate of 0.172 mgm. per cubic centimeter in 5 days while the unfiltered culture produced 0.385 mgm. in the same time (table 2). About one-half of the enzyme producing NH_2 and NH_3 groups had

passed the filter. The other half may have been inside the cells, or may have been adsorbed by the filter.

The protein-sparing action of glucose

Addition of 0.5 per cent of glucose prevented gelatin decomposition almost completely. In 40 days, the increase in amino nitrogen per cubic centimeter was 1.43 mgm. in gelatin, 0.04 mgm. in gelatin with 0.5 per cent glucose. Since the enzyme is partly extracellular, glucose as such cannot affect the action of such enzyme, though it might influence its action within the cell,

TABLE 2

Milligram NH_2 + NH_3 nitrogen in the culture and in its filtrate

AGE OF CULTURE	NH_2 + NH_3 NITROGEN PER CUBIC CENTIMETER	
	In culture	In filtrate
days	*mgm.*	*mgm.*
Start	0.308	
4	0.352	
7	0.514	
9*	0.656	0.656
10	0.735	0.700
11		0.726
12	0.864	0.768
14	1.040	0.828

* Day of filtration.

and especially the formation and secretion of more proteolytic enzyme. Neutralization of the acid in the glucose culture, from pH 6.0 to 7.2, did not result in increased proteolysis. This shows that absence of proteolysis was not due to inhibition of the enzyme by acid, but to the fact that no proteolytic enzyme had been produced by the cells.

A different picture was obtained when the glucose was added to a 7-day-old gelatin culture (fig. 3). Proteolysis continued in both the original culture and the part to which glucose had been added, though the acid formed from glucose retarded the rate of enzyme action. However, even in this culture, the rate of

proteolysis slowly increased as evidenced by the upward trend of the curve. Neutralization from pH 6.2 to 7.2 on the 19th day increased the rate. The culture without glucose reached a pH of 8.3, and when, on the 24th day, this was adjusted to 6.0, proteolysis ceased entirely while it went on a little further in the unchanged part of the culture.

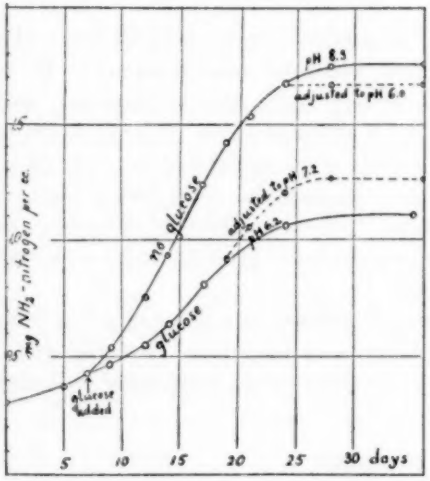

FIG. 3

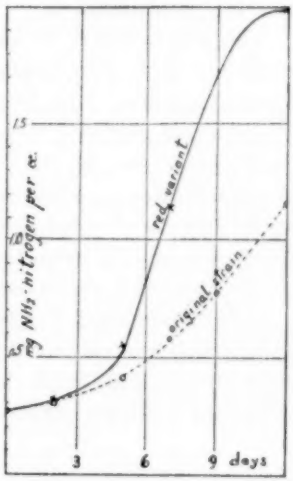

FIG. 4

FIG. 3. NH_2 FORMATION BY BACILLUS SUBTILIS, WITH AND WITHOUT GLUCOSE ADDITION ON THE 5TH DAY, WITH AND WITHOUT pH ADJUSTMENT

FIG. 4. NH_2 FORMATION BY BACILLUS SUBTILIS AND BY ITS ROUGH, RED VARIANT

While addition of glucose at the start prevents formation of the proteolytic enzyme almost entirely, it does not prevent it when added to a 7-day-old culture. Since the cells in the old culture multiply no more, their internal structure cannot be as readily altered as that of young, growing cells. Aside from the direct effect upon the enzyme formation, we have further an inhibition of proteolysis by the acid formed from glucose.

The need of oxygen for gelatin liquefaction could be established, and the redox-potential seemed to play a rôle, but during the quantitative measurement of these factors, a change of the organism occurred.

Dissociation of the culture

The strain used produces a red pigment on glucose media, but growth is colorless without sugar. The new variant, however, producing a water-soluble red pigment in gelatin without sugar, appeared sometimes after 5 days, sometimes not until the 30th day. Transfers from dissociated cultures produced the pigment in 24 to 48 hours.

The red variant is less dependent upon oxygen than the original, and this prevented accurate measurements. It is Gram-variable. The Gram-positive cells appear granular, and the stained particles look like a streptococcus. Liquefaction is very rapid (see fig. 4). Growth is so agglutinative that plate counts or direct counts become impossible. For this reason, it could not be decided whether the early point of inflection in figure 4 is merely caused by a more rapid growth, or by a change of the character of the enzyme.

This change from the white *B. subtilis* to a red strain has been observed frequently in this laboratory by several bacteriologists. It occurs not only with the laboratory strain mentioned, but also with the Marburg strain.

SUMMARY

Gelatin decomposition by *Bacillus subtilis*, as measured by formol titration, is at least partly due to an extracellular enzyme which passes the Berkefeld filter. Therefore, no definite relation exists between the number of cells and the amount of decomposed gelatin.

Addition of glucose at the start prevents almost entirely the formation of the proteolytic enzyme. Addition to a culture in which multiplication of cells has ceased does not prevent the formation of more enzyme though the rate of enzyme action is decreased by the acid formed from glucose.

Bacillus subtilis dissociates at times to a more rough type with red pigment and more intense action on gelatin.

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THE FERMENTATION OF MUCIC ACID BY SOME INTESTINAL BACTERIA

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In the course of a study of the fermentation of some sugar derivatives by bacteria, it was found that various intestinal bacteria showed interesting results with mucic acid. *Escherichia coli*, *Shigella dysenteriae*, Sonne-Duval, *Salmonella enteritidis*, and *S. schottmülleri* fermented this compound with the production of acid. *Aerobacter aerogenes* and *Salmonella aertrycke* produced acid and gas. *Eberthella typhi*, *Shigella dysenteriae*, Flexner, *Salmonella cholerae-suis*, and *Salmonella paratyphi* did not ferment the compound (Sternfeld and Saunders, 1937). Inasmuch as the differentiation of these organisms is of interest it was decided to extend the original observation by testing various strains of each organism.

We are deeply indebted to Dr. P. R. Edwards of the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Dr. S. A. Koser of the Department of Bacteriology, University of Chicago, Miss Virginia Breaks of the Children's Memorial Hospital, Chicago, and Mr. Alfred Noyes of the Coroner's Office, Chicago, for supplying us with the various strains of bacteria used in this study.

Mucic acid, the dicarboxylic acid of galactose, was prepared by the usual method from lactose, the sugar being hydrolyzed and oxidized by treatment with concentrated nitric acid. On cooling, the water-insoluble mucic acid precipitates, and after filtration is recrystallized several times from 50 per cent ethyl alcohol. The pure product is obtained in the form of white crystals, melting at 212° and optically inactive.

For fermentation tests, the acid was neutralized with sodium

hydroxide and added in 0.5 per cent concentration to ordinary nutrient broth, pH 7.0 to 7.2. Brom-cresol-purple (pH range 5.2 to 6.8) was added as indicator and the medium tubed with inner inverted fermentation vials. Sterilization was carried out by autoclaving, using fifteen pounds of steam for ten minutes. It had previously been found that sterilization of the sugar acid by filtration through a Seitz filter yielded results that in no way differed from those obtained by using the ordinary sterilizing procedure. An incubation temperature of 37°C. was employed

TABLE 1

Fermentation of mucic acid

ORGANISM	ACID	ACID AND GAS	NEGATIVE
A. aerogenes (5)	4	1	0
E. coli (5)	3	1	1
E. typhi (9)	0	0	9
S. paratyphi (4)	0	0	4
S. Schottmülleri (4)	3	1	0
S. aertrycke (21)	9	12	0
S. cholerae-suis (7)	0	0	7
S. enteritidis (12)	3	9	0
S. suipestifer (4)	0	0	4
S. dysenteriae, Shiga (2)	0	0	2
S. dysenteriae, Flexner (4)	0	0	4
S. dysenteriae, Sonne-Duval (8)	6	0	2

in all the tests. The controls in each experiment consisted of a non-inoculated tube of medium. In some experiments a control was used consisting of tubes of ordinary nutrient broth (without the sugar acid) inoculated with each organism. In this fashion, acid production due to a spontaneous breakdown of the sugar or due to abnormal bacterial breakdown of the constituents of the nutrient broth could be detected.

Readings were taken once a day for five successive days and then every third day for a total period of two to three weeks. In table 1 are presented the results of the fermentation of mucic acid.

All positive fermentations except two strains of *Shigella*

dysenteriae, Sonne-Duval, occurred within forty-eight hours, many fermenting within twenty-four hours. The two exceptions showed positive fermentation after nine days. An interesting observation was that most of the strains exhibited an alkaline reaction from forty-eight to seventy-two hours after they had shown a positive fermentation, indicating in all probability a further utilization of the intermediary acid products resulting from the breakdown of the mucic acid.

It is of some interest to compare the results obtained by the use of the mucic acid with those obtained when *d*-tartaric acid was used in fermentation tests for differentiation of some types within the *Salmonella* group (Jordan and Harmon, 1928). *S. paratyphi and S. schottmülleri* did not ferment the tartrate, whereas *S. aertrycke, S. enteritidis, S. cholerae-suis, S. abortivo-equinus,* and *S. herschfeldii* produced acid. Koser (1923) showed that *E. coli* and *A. aerogenes* would, in most cases, grow on a synthetic medium where the only source of carbon was mucic acid. Kendall and Gross (1930) reported that mucic acid was not fermented by *B. paratyphosus.*

Barker (1936) showed that *A. aerogenes* would ferment *d*-tartaric, *l*-malic, and fumaric acids but not *d*-malic, maleic, or succinic acids.

SUMMARY

The fermentation of mucic acid by various intestinal bacteria was tested, using eighty-two strains. With the exception of three strains, the results were consistent in that all the strains of one species either fermented or did not ferment this compound. *Aerobacter aerogenes, Escherichia coli, Salmonella schottmülleri, Salmonella aertrycke, Salmonella enteritidis,* and *Shigella dysenteriae,* Sonne-Duval, ferment mucic acid; whereas, *Eberthella typhi, Salmonella paratyphi, Salmonella cholerae-suis, Salmonella suipestifer, Shigella dysenteriae,* Shiga and *Shigella dysenteriae,* Flexner, do not ferment mucic acid. Inasmuch as mucic acid is very readily obtained, we believe that this compound will be of help to the bacteriologist working with intestinal bacteria.

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GELATIN AS A SOURCE OF GROWTH-PROMOTING SUBSTANCES FOR BACTERIA[1]

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In the course of studies on the nutritional requirements of some of the more exacting bacteria it was found that the addition of certain brands of gelatin to a simplified synthetic medium caused growth of a number of bacteria which were unable to develop in the same medium without the gelatin. The use of gelatin in amounts ordinarily employed for culture media produced results which were comparable to, or at times better than, those secured by the use of small amounts of fractionated extracts of spleen, liver or yeast.

Extracts of spleen, liver, yeast and other materials have been subjected by a number of workers to chemical fractionation in attempts to obtain growth-promoting substances responsible for development of the more fastidious bacteria. Since gelatin appeared to offer a promising source of these substances a further study of its effect was made. A comparison of results secured with the use of Difco gelatin and with a typical tissue preparation is given in table 1.

The synthetic medium used in this experiment was similar in composition to that employed in previous work (Koser and Saunders, 1935) and contained inorganic salts, glucose, asparagin, cystine and tryptophane. None of the microorganisms were able to develop in the synthetic medium alone. The amounts of added spleen preparation and of gelatin necessary to secure development of five representative organisms are shown in the

[1] This investigation was aided by a grant to the University of Chicago from the Rockefeller Foundation.

TABLE 1

Comparative growth-promoting effect of a spleen preparation and of gelatin when added to a synthetic medium

	SPLEEN PREPARATION*				GELATIN (DIFCO)			
	mgm. per cc. added	Development of culture, days			mgm. per cc. added	Development of culture, days		
		1	2	4		1	2	4
Staphylococcus albus	None	0	0	0	None	0	0	0
	0.1	+	+++	+++	25.0	++	++++	++++
	0.03	0	+	++	10.0	0	+++	+++
	0.01	0	+?	+	3.0	0	++	+++
					1.0	0	+	++
Streptococcus pyogenes (from scarlet fever)	None	0	0	0	None	0	0	0
	1.0†	+	++	++	50.0	+++	++++	++++
	0.1†	0	0	0	25.0	0	+	++
					10.0	0	0	0
Brucella abortus	None	0	0	0	None	0	0	0
	0.3	+?	++	+++	50.0	+	++	++++
	0.03	0	?	+	25.0	0	+	++
					10.0	0	0	+
Corynebacterium diphtheriae	None	0	0	0	None	0	0	0
	0.1	+	++	+++	25.0	+	+++	+++
	0.01	0	+	++	10.0	+	++	+++
	0.001	0	0	+	1.0	0	+?	++
Shigella paradysenteriae	None	0	0	0	None	0	0	0
	0.1	++	+++	+++	25.0	+++	+++	+++
	0.01	0	+	+	10.0	+	+	+
					3.0	0	0	+

* The spleen preparation had been subjected to a process of fractionation to remove a part of the inert material while preserving the growth-promoting substances.

† The results shown for the streptococcus were secured with crude spleen extract. When similar amounts of partially purified spleen preparation were used there was no growth. Evidently one or more essential substances for this organism are lost in our present method of preparation.

In this experiment the gelatin was autoclaved separately and added to the synthetic medium in the amounts specified.

0 = no visible growth; ? or +? = questionable or very light growth; + to ++++ = light to luxuriant growth.

table. It will be seen that considerably larger quantities of gelatin were required to produce a growth-promoting effect comparable to that resulting from the use of small amounts of spleen preparation. Usually from one hundred to three hundred times as much was needed for this purpose.

A number of other microorganisms, in addition to those listed in the table, were also tested in the gelatin synthetic-medium combination. Among these were four strains of hemolytic streptococci, six of green-producing streptococci from various sources, four strains of meningococci, representatives of types I, II and III pneumococci, four strains of the gonococcus, six strains of *Leuconostoc*, five of *Staphylococcus aureus*, one each of *Brucella abortus* and *Brucella suis*, and the yeast *Saccharomyces cerevisiae*. When 5 per cent Difco gelatin was added to the synthetic medium all of the above mentioned organisms developed. In many cases growth was rapid and a pronounced turbidity was produced within 24 hours at 37°C. In a few cases, however, growth in the gelatin medium was somewhat retarded and scantier in amount. Thus, one of the four strains of meningococcus, a strain of type III pneumococcus, two of the four cultures of the gonococcus and one strain each of *Streptococcus viridans* and *Leuconostoc* gave only a light growth which did not appear until the second to fourth day of incubation.

In the synthetic medium without the gelatin none of the foregoing organisms were able to develop. Transfer of a loopful of synthetic medium to veal-infusion agar slants, blood agar or other appropriate medium at various intervals after inoculation showed that the number of cells gradually decreased until none could be found. This was in decided contrast to the growth in the gelatin tubes. Additional data, showing plate counts of cultures in the gelatin synthetic-medium combination and in the synthetic medium alone, are given in table 2.

Two other organisms were used which, in contrast to the foregoing strains, were able to multiply in the synthetic medium. One of these, *Sarcina lutea*, usually produced marked growth after several days of incubation while the other, *S. paradysenteriae*, Duval-Sonne type, never produced more than a light

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	0.1	+	+++	+++	25.0	++	++++	++++
	0.03	0	+	++	10.0	0	+++	+++
	0.01	0	+?	+	3.0	0	++	+++
					1.0	0	+	++
Streptococcus pyogenes (from scarlet fever)	None	0	0	0	None	0	0	0
	1.0†	+	++	++	50.0	+++	++++	++++
	0.1†	0	0	0	25.0	0	+	++
					10.0	0	0	0
Brucella abortus	None	0	0	0	None	0	0	0
	0.3	+?	++	+++	50.0	+	++	++++
	0.03	0	?	+	25.0	0	+	++
					10.0	0	0	+
Corynebacterium diphtheriae	None	0	0	0	None	0	0	0
	0.1	+	++	+++	25.0	+	+++	+++
	0.01	0	+	++	10.0	+	++	+++
	0.001	0	0	+	1.0	0	+?	++
Shigella paradysenteriae	None	0	0	0	None	0	0	0
	0.1	++	+++	+++	25.0	+++	+++	+++
	0.01	0	+	+	10.0	+	+	+
					3.0	0	0	+

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Two other organisms were used which, in contrast to the foregoing strains, were able to multiply in the synthetic medium. One of these, *Sarcina lutea*, usually produced marked growth after several days of incubation while the other, *S. paradysenteriae*, Duval-Sonne type, never produced more than a light

turbidity in the synthetic medium. These two organisms exhibited a decided contrast to each other with respect to cell multiplication upon the addition of gelatin. Development of

TABLE 2

Comparative numbers as determined by plate counts in synthetic medium with and without 5 per cent gelatin

ORGANISMS USED	TIME INTERVAL	PLATE COUNTS	
		Synthetic medium + 5 per cent gelatin	Synthetic medium without gelatin
	hours	*numbers per cc.*	*numbers per cc.*
Diplococcus pneumoniae (Type I)	0	12,000	12,000
	24	150,000	250
	48	4,200,000	150
Streptococcus pyogenes (scarlet fever)	0	8,500	10,500
	24	55,000	100
	48	2,850,000	75
Streptococcus fecalis	0	9,000	8,000
	24	8,000,000	24,000
	48	200,000,000	2,500
Staphylococcus albus	0	10,000	12,000
	24	200,000,000	4,500
	48	120,000,000	2,400
Corynebacterium diphtheriae	0	300,000	270,000
	24	10,000,000	30,000
	48	50,000,000	2,500
Sarcina lutea	0	4,500	5,000
	24	1,000	110,000
	48	15,000	2,500,000
	96	750,000	1,100,000

Difco gelatin was used for these experiments.

Since many of these organisms occur in chains or clusters, the actual cell population is distinctly greater than the figures given by plate counts.

the Duval-Sonne dysentery organisms was accelerated by the addition of gelatin. The development of *Sarcina lutea*, however, was markedly retarded by amounts of gelatin above two to three per cent.

In further work with the whole series of cultures, the effect of varying amounts of Difco gelatin was tried. It was found that the most pronounced growth-promoting effect was usually secured with 5.0 per cent gelatin. With progressively decreasing quantities slower and scantier development occurred. Certain types, as might be expected, were affected to a greater extent than others when the concentration of gelatin was lowered. In general the hemolytic streptococci, pneumococci, *Brucella* and *Pasteurella* required larger amounts than others and either refused to grow or developed poorly when 1 per cent gelatin was used. With 0.1 per cent gelatin only a few organisms were able to multiply sufficiently to produce a visible turbidity and they developed slowly. In this category were several staphylococci and a strain of the diphtheria bacillus. Concentrations of gelatin over 5 per cent seemed to offer no added advantage and in certain instances 10 per cent and 15 per cent concentrations appeared to be somewhat inhibitory.

In some experiments the gelatin was sterilized separately in the autoclave and added to the medium aseptically, while in others the gelatin was dissolved in the synthetic medium and sterilized with it. While there was no very marked difference in the results secured with these two methods, there was an indication in several different experiments that visible growth appeared a trifle sooner and occurred in slightly lower concentrations of gelatin in those cases where the gelatin was autoclaved in the finished medium.

Comparative tests were made with several different brands of gelatin. A sample of Eastman's photographic gelatin and one of ordinary household gelatin were compared with the Difco product. In this connection it should be mentioned that many gelatins are quite acid and require neutralization when used in culture media. Of these several gelatins, that used for photographic purposes represented the purest product and it is interesting that its growth-promoting effect was decidedly inferior to that exhibited by the two other samples. When the photographic gelatin was used only a few of the microorganisms developed to the point of visible turbidity and they were the

ones which were able to grow when only small amounts (0.1 to 0.3 per cent) of the Difco product were supplied. Interestingly, the development of *Sarcina lutea* was not restrained by the addition of 3 per cent photographic gelatin, whereas it was markedly delayed by similar amounts of the household or Difco products.

Using an optimum amount of Difco gelatin, a number of variations in the composition of the basic synthetic medium were tried. Since the original medium contained only a limited number of amino acids, another more elaborate synthetic medium was substituted for it. The latter contained small quantities of 15 amino acids, in addition to 0.2 per cent glucose and inorganic salts (Koser, Finkle, Dorfman, Gordon and Saunders, 1938). However, the resultant growth after the addition of Difco gelatin was as a rule no more rapid nor luxuriant than with the restricted synthetic medium.

In other experiments certain of the constituents of the synthetic medium were omitted. Omission of the amino acids resulted in little decrease in either the rapidity or luxuriance of development. Apparently the necessary amino acids, as well as other essential substances, were present in the gelatin preparation (Difco). Omission of both the glucose and the amino acids lowered somewhat the nutritive value of the mixture, particularly when smaller amounts of gelatin (0.3 per cent and 1.0 per cent) were used. Omission of the inorganic salts of the synthetic medium produced a culture environment distinctly inferior to that of the complete medium. A solution of gelatin alone permitted growth of only a limited number of the organisms.

On ashing 5-gram samples of both Difco and household gelatin it was found that the factors responsible for growth were destroyed. The ash which remained after ignition, when added to several different synthetic media in appropriate amounts, exerted no growth-promoting effect whatever. There was no detectable ash after ignition of the Eastman gelatin.

The most obvious explanation of the growth-promoting effect shown by several of the brands of gelatin is that essential organic substances of unknown nature are supplied. Since the organisms refused to develop in a synthetic medium containing a wide

assortment of amino acids it may be assumed that the substances responsible for growth are other than the usual amino acids. It is realized, however, that other explanations should be considered. Certain inorganic salts, in addition to those supplied in the synthetic medium, may be present in the gelatin and may be required in addition to organic compounds. The effect, too, might be interpreted as being due to combination of the gelatin with inhibitory amounts of metallic impurities in the medium, or to an alteration in the viscosity or the surface tension of the culture solution from a less to a more favorable level. Since the gelatin used for photographic purposes is supposedly a purer product than other gelatins, one is led to assume that the substances needed by the organisms are present as impurities. It must not be overlooked, however, that some brands of gelatin may contain substances which restrain development and such an effect might lead to the conclusion that growth factors were absent.

It seemed of interest to compare the viscosity of several gelatins which gave quite different results in the tests of growth-promoting activity. To measure viscosity of the gelatin-synthetic medium combination, one cubic centimeter was allowed to flow through a bulb and capillary arrangement and the time was noted. All measurements were made at 40°C. Distilled water was used as the standard. The synthetic medium without gelatin gave results similar to those secured with distilled water. The following readings are typical:

Distilled water	1 minute 5.8 seconds
5.0 per cent photographic gelatin in synthetic medium	6 minutes 12.7 seconds
5.0 per cent Difco gelatin in synthetic medium	6 minutes 11.6 seconds

It is evident that the photographic gelatin and the Difco product gave similar viscosity readings, though the growth-promoting effects were quite different when a 5.0 per cent concentration was incorporated in the synthetic media.

If the effect of the gelatins may be attributed to the presence of certain organic impurities, the nature of these substances becomes

a matter of immediate importance since such exacting types as hemolytic streptococci from scarlet fever and pneumococci were able to develop. Recent work on the growth requirements of microorganisms has thrown some light on the needs of several types, especially with respect to substances other than the ordinary amino acids. From the work of Schopfer (1935), Tatum, Wood and Peterson (1936), Williams and Rohrman (1936), Miller (1936), Knight (1937), Mueller (1937), Mueller and Cohen (1937), and Koser, Finkle, Dorfman, Gordon and Saunders (1938) it is evident that vitamine B_1, beta alanine, and nicotinic acid, either singly or in certain combinations, are needed for the development of propionic acid bacteria, staphylococci, the diphtheria bacillus, several strains of yeast and a mold.

Whether the foregoing substances are present in the brands of gelatin exerting a growth-promoting effect has not been determined. However, since a number of the organisms used in our gelatin experiments refused to develop in synthetic media even after the addition of the three foregoing substances, it would appear that other compounds yet to be identified are necessary. This was true especially of the hemolytic streptococci, pneumococci and allied types. The several brands of commercial gelatin constitute a promising source of material for further studies on the extraction and purification of substances needed by these organisms. That such growth-promoting substances seem to be present in several gelatins in considerably smaller quantities than in tissue infusions is not necessarily a disadvantage, since it might be possible to separate them more readily from gelatin than from such sources as liver, spleen and yeast. Further work alone these lines is now in progress.

SUMMARY

Some brands of gelatin exert a growth-promoting effect upon a number of the more exacting bacteria. This effect appears to be similar to that caused by fractions of spleen, liver and yeast and may be demonstrated by the same method. Considerably larger quantities of gelatin, however, are needed to produce an equivalent effect upon culture development.

A purified gelatin, prepared for photographic purposes, showed relatively little growth-promoting effect.

Certain commercial brands of gelatin appear to constitute a promising source of substances essential for the growth of organisms such as streptococci, pneumococci, dysentery bacilli, *Brucella* and other types which refuse to develop in the usual synthetic media.

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INFLUENCE OF pH ON THE DISSIMILATION OF GLUCOSE BY AEROBACTER INDOLOGENES[1]

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Osburn, Brown and Werkman (1937) observed a critical pH level in the dissimilation of glucose by *Clostridium butylicum* near 6.3. Above this level butyl alcohol and isopropyl alcohol were not formed. The significance of this critical pH was attributed to the fact that in mixtures of acetic (or butyric) acid and its sodium salt below 6.3 there will be "free" acid, i.e., acid which can be distilled from solution and can be converted into alcohols by *C. butylicum*.

This concept of critical pH is applied to the dissimilation of glucose by *Aerobacter indologenes* in the present investigation, particularly with reference to the findings of Reynolds and Werkman (1937), that when sufficient acetic acid is present in a glucose dissimilation by *A. indologenes*, no gaseous hydrogen is evolved and the acetic acid disappearing in the medium is accounted for as 2,3-butylene glycol + acetylmethylcarbinol. Their conclusions were to the effect that acetic acid occurring in the fermentation was transformed into 2,3-butylene glycol + acetylmethylcarbinol.

The principle of critical pH probably has a general application to cellular metabolism. Although pH 6.3 was found to be the critical level in the case of *C. butylicum* in converting butyric and acetic acids into corresponding alcohols, it is likely that each organism will show several such levels which will be determined by the dissociation of the substance subject to change. Furthermore, as pointed out by Osburn and Werkman (1937), the change

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in metabolism does not occur sharply at pH 6.3, but the effect is gradual and extends over a relatively narrow range.

The dissimilation of glucose by *A. indologenes* at pH levels below and above 6.3 has been studied in the present work. The results are of particular significance for an understanding of bacterial dissimilation as well as having certain practical implications in procedures involving the presence of acetylmethylcarbinol. In the latter respect, reference may be made to the Voges-Proskauer test and the formation of acetylmethylcarbinol in butter cultures.

In the work of Reynolds and Werkman (1937) no mention of a critical pH level at which the mechanism of dissimilation changes its nature, is made. In the present investigation the significance of levels above and below pH 6.3 is shown in the dissimilation of glucose by *A. indologenes*. The results indicate that the gas ratio of carbon dioxide to hydrogen, the occurrence of acetylmethylcarbinol and 2,3-butylene glycol and the amounts of acetic and formic acids are dependent on the pH of the fermentation and that the range near pH 6.3 is a critical level; below this range, the course of dissimilation is markedly different from that above.

METHODS

Glucose plus acetate fermentations were conducted in 4-liter Erlenmeyer flasks fitted with proper openings for removal of gas, taking of samples, and addition of alkali. Brom-thymol-blue was used as an indicator and the pH maintained by adding normal sodium hydroxide.

The medium consisted of glucose 2 per cent, ammonium sulfate 0.3 per cent, peptone 0.5 per cent, sodium acetate 0.6 per cent and 0.1 M dipotassium phosphate. It is of interest that distilled water was not suitable for making up the medium. Tap water provided excellent growth. When Speakman's salts in distilled water replaced the tap water, growth was not nearly as good. When tap water was evaporated and the residue ashed, addition of the latter provided growth equal to that in the tap water medium. Apparently the essential constituent is inorganic.

The pH was adjusted with sulfuric acid and the medium sterilized at 20 pounds pressure for 30 minutes. Glucose was sterilized separately. Incubation was at 30°C.

Carbon dioxide was collected in Bowen potash bulbs in a drying train. Residual carbon dioxide was determined on a sample collected in alkali which was acidified and refluxed in a stream of CO_2-free air. The carbon dioxide was collected and weighed in a Bowen bulb.

Hydrogen was determined by continuous combustion over heated copper oxide and weighing the water formed. Oxygen-free nitrogen was continuously led through the flasks to remove gases.

An aliquot part of the fermented medium was made acid to congo red and distilled to half volume. The distillate was neutralized to phenolphthalein, again distilled and the alcohol determined on this distillate. The two residues were combined and steam distilled to recover the volatile acids. Total volatile acidity was determined on this distillate by titration. The formic acid was determined according to Auerbach and Zeglin (1922), and the acetic acid obtained by difference. In the presence of acetylmethylcarbinol it was necessary to neutralize the volatile acid distillate, evaporate to small volume and remove at least six volumes by alkaline steam distillation to avoid interference with the determination of formic acid.

Ethyl alcohol was determined on an aliquot part of the neutral volatile fraction by oxidation with a sulphuric acid-potassium dichromate mixture in a closed flask on the steam bath. The mixture was then steam distilled and the acetic acid determined. The alcohol determination was corrected for the acetic acid originating from the acetylmethylcarbinol.

Acetylmethylcarbinol was determined on an aliquot part of the fermented medium according to Stahly and Werkman (1935).

2,3-Butylene glycol was steam distilled from a sample of the medium from which the sugar had been removed by the copper-lime technique of Hewitt (1931); 13 volumes or more were collected from a constant volume of 20 ml. which contained 25 grams of $MgSO_4 \cdot 7H_2O$. Butylene glycol was determined on an

aliquot part of the distillate by oxidation with periodic acid and directly distilling the acetaldehyde into a solution of sodium bisulphite. The aldehyde was determined by titration with iodine.

The lactic acid was determined on the residue according to Friedemann and Graesser (1933).

The experiments with cell suspensions were conducted in Erlenmeyer flasks containing 300 ml. of 0.1 M phosphate with substrate, adjusted to the desired pH. Carbon dioxide and hydrogen were collected in bottles containing alkali; the former was absorbed and determined on an aliquot part, the latter was determined by volume displacement. The hydrogen evolved was in contact with the medium and was available for reduction of the acetic acid. The inoculum consisted of about 5 grams of cell paste per 300 ml. of medium. Air was displaced from the flasks with nitrogen when the fermentations were started. After four days at 30°C. the flasks were removed and contents analyzed according to the procedure used in the glucose ferementations.

EXPERIMENTAL

In tables 1 and 2 are given results of typical experiments in which the influence of pH on the mechanism of dissimilation of glucose by *A. indologenes* is shown. In the fermentation kept above pH 6.3 (near 7.0) the yields of carbon dioxide and 2,3-butylene glycol are greatly suppressed as compared with an acid fermentation. Acetic and formic acids accumulate and added acetic acid is not attacked. The quantitative relationships of the products are quite different from those in a dissimilation occurring below pH 6.3. Here, acetic acid, even that added, disappears rapidly with the simultaneous occurrence of the 4-carbon compounds, acetylmethylcarbinol and 2,3-butylene glycol (fig. 1); also the production of carbon dioxide is large. Whether the failure of acetic acid to act as an intermediate in alkaline fermentation is due only to a lack of free acid or a dearth of available hydrogen or both, is not clear. Should the presence of formic acid be the result of a synthesis from carbon dioxide and hydrogen, the failure of acetic acid reduction probably results from the utilization of hydrogen in the reduction of carbon

TABLE 1

Fermentation of glucose plus added acetic acid by Aerobacter indologenes. pH maintained above 6.3

TIME	SUGAR FERMENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYLCARBINOL	2,3-BUTYLENEGLYCOL	ETHYL ALCOHOL	2,3-BUTYLENEGLYCOL + ACETYLMETHYLCARBINOL	CARBON RECOVERY	O/R INDEX
hours											*per cent*	
0	0				25.77							
16	19.91	15.03		19.15	37.65	2.72	0.24	9.35	15.20	9.59	108	0.89
20	50.90	33.90		49.90	47.70	2.76	1.27	12.50	36.70	13.77	89	1.06
23½	89.30	51.00		74.00	96.05	7.10	1.50	15.60	64.60	17.10	92	0.97
31½	113.10	58.5	2.26	95.00	109.00	5.85	0	22.40	74.80	22.40	86.3	0.97
75	113.10	60.24	9.26	95.00	109.10	6.35	0	22.30	75.60	22.30	87	0.95

Products in millimoles per liter.

TABLE 2

Fermentation of glucose plus added acetic acid by Aerobacter indologenes at pH 6.3 or below

TIME	SUGAR FERMENTED	CO_2	H_2	FORMIC ACID	ACETIC ACID	LACTIC ACID	ACETYLMETHYLCARBINOL	2,3-BUTYLENEGLYCOL	ETHYL ALCOHOL	2,3-BUTYLENEGLYCOL + ACETYLMETHYLCARBINOL	CARBON RECOVERY	O/R INDEX
hours											*per cent*	
0	0				24.66							
24	35.93	59.59		10.40	11.84		3.64	31.40	20.8	35.04	99	0.92
29	48.73	74.57		10.20	7.00	4.76	4.30	42.00	27.16	46.30	102	0.89
33	59.13	86.52		10.08	4.80	2.28	4.64	45.50	31.46	50.14	95	0.88
48	96.98	132.69	1.24	19.72	5.31	2.20	2.20	70.00	51.54	72.20	90	0.87
96	111.3	215.66	17.69	12.20	3.35	2.46	1.05	82.00	58.40	83.05	96	1.12
168	111.3	225.66	29.07	1.35	4.95	1.78	2.36	82.20	55.80	84.56	96.5	1.15
264	111.3	230.74	30.83	1.26	6.00	2.70	0.72	87.20	60.02	87.92	100	1.14

Products in millimoles per liter.

dioxide rather than acetic acid. It is to be noted that there was some carbon dioxide and glycol formed in the alkaline fermentation, probably owing to the difficulty of holding the medium alkaline as at times it became sufficiently acid to allow the formation of these products. In subsequent experiments conducted at pH 7.0 or above, the formation of 2,3-butylene glycol was diminished to a still greater extent—from a normal of approximately 70 mM to 2 mM or less. The accumulation of acetic

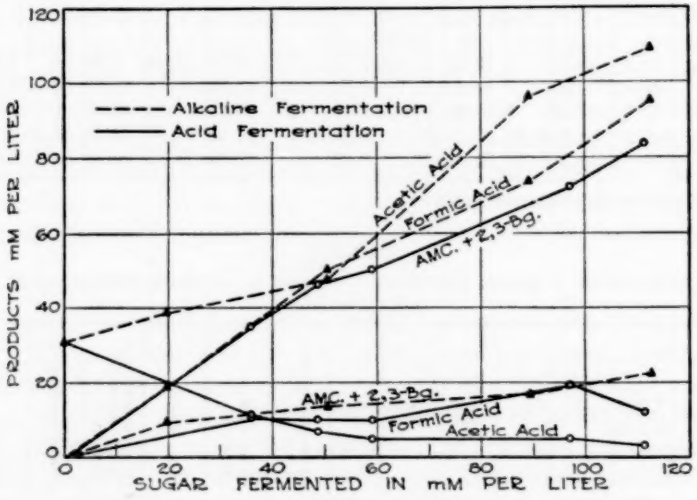

FIG. 1.

acid demonstrates its necessity in an available form for the formation of acetylmethylcarbinol and 2,3-butylene glycol. Incomplete carbon recoveries with the alkaline fermentations suggest that some undetermined product is formed.

It is interesting to note the influence of pH on the gas ratio which varies within large limits. When the pH is maintained below 6.3 and sufficient acetate added, the production of hydrogen is completely suppressed and the carbon dioxide formation is large, whereas, in an alkaline medium the production of both

carbon dioxide and hydrogen can be practically prevented. Under the usual conditions of glucose dissimilation the pH drops within the vicinity of 6.0–5.5 but insufficient acetic acid is present to prevent the liberation of hydrogen and a H_2:CO_2 ratio of 0.3–0.5 is obtained.

As the fermentation nears completion, the pH shows a reversion owing to the conversion of the acetate ion into neutral compounds.

In regard to the source of hydrogen used in the reduction of acetic acid, it is possible that this is furnished by the dehydrogenation of formic acid. However, using heavy cell suspensions, it was impossible to demonstrate the conversion of acetic acid to acetylmethylcarbinol or 2,3-butylene glycol in the presence of formic acid as H_2-donator (table 3). The fermentations were

TABLE 3

Fermentation of acetic and formic acids by cell suspensions of Aerobacter indologenes in acid and alkaline medium

REACTION OF MEDIUM	ACETIC ACID ADDED	FORMIC ACID ADDED	ACETIC ACID	FORMIC ACID	CARBON DIOXIDE	HYDROGEN	FORMIC ACID FERMENTED
pH 6.0–6.3	112.25	137.76	112.35	72.85	62.20	60.56	64.91
pH 7.0–7.3	111.53	136.90	113.30	121.16	12.40	13.38	15.74

Voges-Proskauer test negative. Products in millimoles per liter.

conducted at pH levels of substantially 6.0–6.2 and at 7.0. It will be observed that the acetic acid was recovered quantitatively in each case and that the formic acid broken down was accounted for quantitatively by equimolar amounts of carbon dioxide and hydrogen. It is also of significance that the formic acid decomposed was about four times greater in an acid than alkaline medium. This finding supports in part, the results from a glucose fermentation under the same conditions. The hydrogen from formic acid did not reduce acetic acid under these conditions. However, in the presence of glucose plus acetic acid the yield of hydrogen is greatly suppressed. If formic acid is the only hydrogen-yielding intermediate it is difficult to account for this behavior which suggests that hydrogen from some other source

is necessary for the conversion of acetic acid to acetylmethylcarbinol. Formic acid hydrogen is able to reduce acetylmethylcarbinol to 2,3-butylene glycol by *Escherichia coli* (table 4).

The effect of pH on the action of the formic hydrogenlyase is important. Krebs (1937) found that *E. coli* did not form lactic acid from pyruvic acid anaerobically when the pH was between 6.6–8.0. However, if the pH was lowered to 5.4, considerable lactic acid and carbon dioxide were produced. In an alkaline medium formic and acetic acids were the only final products of pyruvic breakdown. Woods (1936) found in the case of *E. coli* that an alkaline reaction is necessary for a synthesis of formic acid from carbon dioxide and hydrogen. We have

TABLE 4

Reduction of acetylmethylcarbinol by cell suspensions of Escherichia coli and formic acid

	ACETYL-METHYL-CARBINOL	CARBON DIOXIDE	HYDRO-GEN	FORMIC ACID	ACETIC ACID	2,3-BU-TYLENE-GLYCOL	CARBON RECOV-ERY
							per cent
Initial	54.25			112.77			
Final	0	76.31	30.10	39.69	25.73	41.91	102

Products in millimoles per liter.

found that formate in an acid medium is decomposed vigorously. However, at alkaline levels the conditions are relatively more favorable for the synthesis of formic acid than its breakdown. Since formic hydrogenlyase is a reversible system, the retention of carbon dioxide in the medium may tend to drive the reaction toward the synthesis of formate.

SUMMARY AND CONCLUSIONS

A critical pH level has been shown to exist in the dissimilation of glucose by *A. indologenes* in the region of 6.3. Fermentation carried out above this level results in an accumulation of acetic and formic acids. The production of hydrogen and carbon dioxide is greatly suppressed and the formation of acetylmethyl-

carbinol and 2,3-butylene glycol may be prevented if care is taken not to allow the pH to drop within the vicinity of 6.3. When the fermentation occurs below pH 6.3, the acetic acid is converted into acetylmethylcarbinol and 2,3-butylene glycol. If sufficient acetic acid is added to an acid fermentation, the production of gaseous hydrogen is prevented.

It is suggested that the acidity and alkalinity of the medium expresses itself by determining the relative hydrogen accepting ability of acetic acid and carbon dioxide. When the medium is alkaline, carbon dioxide is the better hydrogen acceptor resulting in the formation of formic acid; under acid conditions (approximately 6.3 or less) carbon dioxide cannot compete with acetic acid and acetylmethylcarbinol and 2,3-butylene glycol are formed.

For the conversion of acetic acid into neutral compounds, it is important to have both free acetic acid and available hydrogen. Evidence suggests that the general occurrence of formic acid among the final products of bacterial dissimilation of glucose may be the result of a synthesis from carbon dioxide and hydrogen, a fact which would have important implications in formulating schemes of dissimilation particularly with reference to the breakdown of pyruvic acid into formic and acetic acids or acetaldehyde and carbon dioxide.

The results point clearly to the fact that the gas ratio of (H_2:CO_2) 0.5 is fortuitous. This generally accepted ratio of 0.5 is the result of the conditions under which the determination is made and may vary within wide limits. Review of the literature dealing with quantitative experiments shows that the ratio is usually near 0.3, indicating a marked utilization of hydrogen for the reduction of acetic acid to 2,3-butylene glycol.

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THE FERMENTATION OF DISACCHARIDES BY STREPTOCOCCUS THERMOPHILUS

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Orla-Jensen (1919) was the first to differentiate and describe *Streptococcus thermophilus* as a distinct streptococcal species. Among the unique characteristics of this organism which were pointed out by Orla-Jensen are its general inability to ferment maltose, and its ability to ferment sucrose and lactose with especial vigor, the latter disaccharides being even more favorable to the growth of this organism than are the simple monosaccharides. Orla-Jensen also noted the very interesting fact that *Streptococcus thermophilus* does not ferment mannose, or ferments it very feebly.

Wright (1936) has also noted that some strains of *Streptococcus thermophilus* ferment lactose and sucrose more rapidly than they do the constituent monosaccharides, and on the basis of these results he suggests that *Streptococcus thermophilus* ferments the disaccharides without preliminary hydrolysis. In times past it was a common belief that the lactic-acid bacteria caused a direct fermentation of the disaccharides, since the presence of monosaccharides cannot usually be demonstrated in fermenting cultures by chemical means; even then, however, most investigators held to the theory that the disaccharides were hydrolyzed to the constituent monosaccharides as the first step in their fermentation.

Some years ago one of us became interested in this problem in connection with *Streptococcus lactis*. It was thought that although the preliminary hydrolysis of the disaccharides was not easily proved by chemical means, an indication that such hy-

drolysis actually occurs might be demonstrated by the use of bacteria. It was found that if a culture of *Streptococcus lactis* (sucrose +) and a culture of *Bacterium coli* (sucrose −) are grown together in sucrose broth, gas is formed, though neither of these organisms alone can produce gas from sucrose. Also, when *Streptococcus lactis* (lactose +) and *Proteus* (lactose −) are grown together in lactose broth, gas is likewise produced. At that time it did not appear that this fact had been demonstrated with *Streptococcus lactis*. However, our results were not published, because a survey of the literature showed that such phenomena had been encountered in connection with "false presumptive tests" for *Bacterium coli* in water, in which cases the gas produced from lactose appeared to result from the cooperative action of such organisms as *Streptococcus fecalis*, which can ferment lactose without gas production, and other bacteria capable of producing gas from glucose though not attacking lactose. These facts naturally suggested themselves in connection with Wright's idea concerning the direct fermentation of disaccharides by *Streptococcus thermophilus*.

That *Streptococcus thermophilus* appears to utilize sucrose and lactose more readily than the monosaccharides is easily demonstrated by the rate of acid production in media containing these sugars. Table 1 shows the relative rates of fall in pH with two cultures of *Streptococcus thermophilus* in broths containing 1 per cent of the respective sugars together with 0.3 per cent each of beef extract, peptone and tryptone. Galactose, not included in the table, was fermented more slowly than glucose, as Wright has previously shown.

Streptococcus thermophilus and *Bacterium coli* were grown separately and together in sucrose broth. Since the strain of *Bacterium coli* used could not ferment sucrose, neither of these organisms alone is able to produce gas from this sugar whereas gas production resulted when the two organisms were grown together. The results obtained are shown in table 2.

Experiments were also conducted with *Streptococcus thermophilus* and *Proteus* (lactose −) in lactose broth. The findings (table 3) are in agreement with those obtained with sucrose.

Tests similar to those reported in tables 2 and 3 were conducted with two strains of *Streptococcus thermophilus* and the experiments were repeated a number of times in media of different composition.

TABLE 1

The rate of fermentation (pH) of hexoses and disaccharides by Streptococcus thermophilus

CULTURE	HOURS	GLUCOSE	FRUCTOSE	SUCROSE	LACTOSE
2	0	6.6	6.6	6.6	6.6
	24	6.4	6.2	5.2	5.3
	66	6.0	4.4	4.7	4.3
	168	5.0	4.4	4.5	4.2
6	0	6.6	6.6	6.6	6.6
	24	6.5	6.2	5.3	5.4
	66	6.0	4.4	4.7	4.3
	168	5.0	4.4	4.5	4.2

TABLE 2

Fermentation of sucrose by Streptococcus thermophilus and Bacterium coli

INOCULUM	ACID	GAS
S. thermophilus	+	−
Bact. coli	−	−
S. thermophilus + *Bact. coli*	+	+

TABLE 3

Fermentation of lactose by Streptococcus thermophilus and Proteus

INOCULUM	ACID	GAS
S. thermophilus	+	−
Proteus	−	−
S. thermophilus + *Proteus*	+	+

The formation of gas was revealed by the use of test tubes of broth with vaseline seals, but in some tests it was also demonstrated with the use of inverted Durham tubes.

To repeat these results, a little attention should be paid to the

composition of the medium. In a lightly buffered medium, if *Streptococcus thermophilus* gets a rapid start the associated organisms may be inhibited before visible evidence of gas is obtained. On account of the extreme sensitiveness of *Streptococcus thermophilus* to salt (Sherman, 1937), it may not do well in media heavily buffered with phosphates. We have found a suitable medium to be one containing 1 per cent of the test sugar with 0.3 per cent each of beef extract, peptone, tryptone, and dibasic potassium phosphate, though several other media have been used successfully.

Although the name *Streptococcus thermophilus* has been loosely used by some workers for various heat-tolerant streptococci, this organism has a very peculiar combination of characteristics which widely separates it from the other clearly defined streptococcal types. In our own work covering a considerable experience in addition to our published data (Sherman and Stark, 1931), the various strains studied have made up a remarkably homogeneous group, though somewhat greater variation has been noted by others who have undoubtedly dealt with the true *Streptococcus thermophilus*.

With reference to the fermentation of disaccharides by *Streptococcus thermophilus*, these simple experiments should not be accepted as offering definite proof; they do, however, indicate that this organism probably hydrolyzes the disaccharides to their constituent monosaccharides as the initial step in the fermentation; they do, also, put the burden of proof on those who hold the opposite view.

SUMMARY

It is shown that if *Streptococcus thermophilus* (sucrose +) and *Bacterium coli* (sucrose −) are grown together in sucrose broth, gas is produced, whereas neither of these organisms alone can produce gas from sucrose. Likewise, gas is produced in lactose broth when *Streptococcus thermophilus* (lactose +) and *Proteus* (lactose −) are grown together.

These results suggest, in opposition to one view which has been held in the past, that *Streptococcus thermophilus* probably hy-

drolyzes the disaccharides to their constituent monosaccharides in the fermentation of such sugars.

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INFLUENCE OF TIME AND TEMPERATURE OF INCUBATION ON HEAT RESISTANCE OF ESCHERICHIA COLI[1]

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Field and laboratory observations (Frazier *et al.* 1935) long have indicated that the temperature of incubation of Swiss cheese starter cultures significantly influences their ability to develop following the rather severe heat exposure to which they are subjected during manufacture. If it were true that the temperature of growth had an effect on heat resistance of bacteria, this fact would be of significance in various fermentations, both commercial and natural, and would prove of general interest from the standpoint of the physiology of bacteria. Therefore, an investigation was initiated to determine the influence of incubation temperature and time on the thermal resistance of certain Swiss cheese starter cultures. For the purpose of comparison, similar studies were undertaken with a typical strain (H-52) of *Escherichia coli*. The results of the investigations on *E. coli* are presented in this paper.

Because it produced less acid, was able to develop under a wider variety of environmental conditions, and could be counted fairly accurately by the plate method, *E. coli* gave results which were more conclusive than those obtained with the lactic starter cultures and indicated that *E. coli*, a favorite subject for experimentation, is an ideal organism for studies on the heat resistance of vegetative cells.

Despite the apparent importance of the relationship between the growth temperature and thermal resistance of bacterial cells,

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few attempts have been made to correlate them. That the growth temperature may directly influence the thermal relationships of protozoa has been demonstrated by Dallinger (1887), who by gradually increasing the incubation temperature of three flagellates over a seven-year period raised their maximum temperature from 23° to 70°C. At the same time the optimum and minimum temperatures were raised to such a degree that when cells growing at 70°C. were placed at 15°C. they perished.

Sherman and Cameron (1933) found that when young cultures of *E. coli*, grown at 45°C., were placed in sterile media at 10°C., there was a far greater destruction of cells than when young cultures grown at 10°C. were placed in sterile media at 45°C. The same investigators reported (1934) that young cells of *E. coli* from cultures growing slowly exhibited greater resistance to various deleterious factors than did the cells from cultures growing rapidly. The growth rate was reduced by using low incubation temperatures, dilute media or media of increased osmotic pressure.

Frazier and coworkers (1935) demonstrated that a slight elevation in incubation temperature from 35–37° to 38–39°C. markedly increased the ability of *Lactobacillus helveticus* to develop at high temperatures.

According to Anderson and Meanwell (1936) a thermoduric streptococcus in the lag and early logarithmic phases of growth showed increased resistance to heat when the incubation temperature was reduced below the optimum.

Dorner and Thöni (1936) found that after cells of *Bacterium acidi-propionici* had reached the mature, heat resistant stage, there was little difference between the thermal resistance of cells grown at 22° and at 30°C.

Claydon (1937) reported that at 10°C. *Streptococcus lactis* cultures grew more slowly than at higher temperatures, but attained greater thermal resistance.

Theophilus (1935) demonstrated that spores formed at the optimum temperature for growth were definitely more heat resistant than those formed at temperatures either below or above the optimum.

The influence of age of bacterial cells on their thermal resistance has been more widely studied.

Numerous investigators including Reichenbach (1911), Schultz and Ritz (1910), Sherman and Albus (1923, 1924), Behrens (1923), Hückel (1926), Ørskov (1925), Darányi (1927), Gates (1929), Jensen (1928), Robertson (1927, 1928), Stark and Stark (1929, a, b), Sherman and Stark (1929), Fabian and Coulter (1930), Hammer and Hussong (1931), Frazier and Wing (1931), Heiberg (1932), Dorner and Thöni (1936), and Claydon (1937) have demonstrated by various methods that young cells are far more susceptible to adverse environmental influences than are older, more mature bacterial cells.

EXPERIMENTAL

In order to maintain the most uniform conditions possible during these investigations, all cultures were incubated in thermostatically controlled water baths, and the time and temperature of incubation were carefully controlled. Moreover, all cultures were carried in freshly prepared, sterile reconstituted skim milk always prepared from the same lot of skim milk powder, and, unless otherwise indicated, one per cent of inoculum was used. Stock cultures, transferred weekly, were incubated at 37°C. for 24 hours after which they were kept at 10°C. until the time for the next transfer.

When it was necessary to inoculate cultures for studies of heat resistance, an inoculating culture was prepared by transfer of one per cent of inoculum from the most recent stock or mother culture to a six-ounce bottle containing 100 cc. of milk. Unless otherwise indicated, this bottle was then incubated under the same conditions as the stock or mother culture and, following incubation, was placed at 10°C. for 36 hours. Then, at varying intervals, inoculations were made from the inoculating culture into triplicate tubes, each containing 10 cc. of milk. The tubes were incubated for the periods and at the temperatures desired. When the triplicate tube cultures were of the required age, they were removed from the water bath. One cubic centimeter of culture from each of the triplicates was placed in a test tube

containing 3 cc. of sterile 2 per cent sodium citrate. The contents were thoroughly mixed; 0.1 cc. was transferred to a tube containing 10 cc. of milk at a temperature of 10°C.; and a sample was removed for the plate count. The tubes were then placed in a mechanically stirred water bath at a temperature of 54°C.; one minute was allowed for the temperature rise, and then heat treatment was carried out at 53°C. for 30 minutes, after which treatment the tubes were immediately cooled and samples removed for plate counts. Plain nutrient agar was used, and plates were incubated at 37°C. for 48 hours.

Curran and Evans (1937) have shown that the indicated percentage survival of cells during heat treatment may be greater if some medium is employed which is superior to plain nutrient agar. Addition of a fermentable carbohydrate to the agar was undesirable because of the resulting gas formation. The results obtained with plain nutrient agar were very uniform and it appeared to be most suitable for these particular studies.

When larger samples were desired, one-fourth per cent of the culture to be heat shocked was transferred to Erlenmeyer flasks containing 450 cc. of milk. The temperature of the milk in the flasks was raised to 53°C. in a period of about five minutes, maintained at 53°C. for 30 minutes and then lowered. Plate counts were made before and after heat treatment.

Percentage survival of mature cells grown at various temperatures

In a preliminary experiment, inoculating cultures were prepared from stock cultures, incubated, respectively, at 28°, 30°, 30.5°, 38.5° and 40°C. for varying periods, and then transfers were made from the inoculating cultures to flasks of milk. The inoculated flasks were heated at 53°C. for 30 minutes, and the heat resistance of the respective cultures was determined. Table 1 shows the percentage survival during heat treatment of cultures which had been incubated for varying periods at the six different temperatures.

These results demonstrate that during the maximum stationary phase of growth the maximum percentage survival of cells at 38.5° and 40°C. is distinctly greater than that obtained at lower

temperatures such as 28°, 30° and 30.5°C. Heat resistance appears to be lowest at 28°C., the lowest incubation temperature employed.

TABLE 1

Heat resistance of Escherichia coli grown at different temperatures for varying periods and then heat shocked at 58°C. for thirty minutes

TEMPERATURE OF INCUBATION	TIME OF INCUBATION	PLATE COUNT		PERCENTAGE SURVIVAL
		Before heating	After heating	
°C.	hours	nos. per cc.	nos. per cc.	
28	38	1,467,000	120,000	8.2
	42	1,445,000	120,000	8.3
	48	1,540,000	93,000	6.0
	54	1,447,000	113,000	7.8
	60	1,333,000	107,000	8.0
30	37	1,674,000	577,000	34
	42	1,393,000	383,000	28
	46	1,233,000	377,000	31
	51	1,357,000	320,000	24
30.5	42	1,510,000	427,000	28
	48	1,390,000	300,000	22
	54	1,257,000	367,000	29
38.5	21	1,341,000	875,000	65
	24	1,188,000	948,000	79
	27	1,145,000	952,000	83
40	10	1,413,000	267,000	19
	12	1,480,000	533,000	36
	16	1,623,000	753,000	46
	20	1,143,000	827,000	72
	24	1,352,000	770,000	57

Heat resistance of cultures of Escherichia coli carried continuously for two weeks at 28° and 38.5°C., respectively

The results shown in table 2 indicate the heat resistance of cultures of *E. coli* after only one incubation period at various temperatures. However, it was considered possible that the heat resistance of a culture might be altered by numerous successive transfers at a definite temperature. Therefore, mother

cultures were inoculated from the stock cultures and carried in tubes containing 10 cc. of milk. According to the results of the previous experiment, shown in table 1, the maximum heat resistance at 28° was maintained from at least the 38th to the 60th hour, and at 38.5° it occurred around the 24th to the 30th hour. In order to make the transfers at the two temperatures at about the time of maximum heat resistance, the 28° culture was transferred every 48 hours and the 38.5° culture every 24 hours. Heat resistance determinations were made after the first transfer and after two weeks of successive transfers at the two temperatures.

TABLE 2

Heat resistance of cultures of Escherichia coli carried at 28° and 38.5°C., respectively, and heat shocked at 53°C. for thirty minutes

INCUBATION OF MOTHER CULTURE		NUMBER OF TRANSFERS	PLATE COUNT		PERCENTAGE SURVIVAL
Temperature	Time		Before heating	After heating	
degrees	*hours*		*nos. per cc.*	*nos. per. cc.*	
28	48	1	1,133,000	253,000	22.3
		1	1,327,000	130,000	9.9
38.5	24	1	1,540,000	960,000	62.3
		1	1,417,000	1,083,000	76.4
28	48	7	1,400,000	250,000	17.9
		7	1,570,000	160,000	10.2
38.5	24	14	1,250,000	1,040,000	83.2
		14	1,490,000	1,210,000	81.2

The cultures to be heated were transferred to flasks of sterile milk and then heat shocked at 53°C. for 30 minutes. Plate counts were made before and after heating. According to the results shown in table 2, the maximum resistance at 28° is again far lower than at 38.5° after both one and numerous transfers at the two temperatures. There seems to be little doubt that when *E. coli* is grown under the conditions of this experiment, thermal resistance during the maximum stationary phase, the time when a culture is considered by most workers to be at the peak of its heat resistance, is far greater when a higher incubation tempera-

ture is used. The results further suggested that a slight increase in heat resistance occurred as a result of numerous successive transfers at the higher temperature.

Influence of age of cells and incubation temperature on heat resistance of E. coli

Next a study was made of the progressive changes taking place in the heat resistance of cultures grown at 28° and 38.5°C. during periods varying from the time of inoculation to the end of the

TABLE 3

Heat resistance of cultures of Escherichia coli grown at 28°C. for varying periods and then heat shocked at 53°C. for thirty minutes

AGE OF CULTURE	PLATE COUNT		PERCENTAGE SURVIVAL
	Before heating	After heating	
hours	*nos. per cc.*	*nos. per cc.*	
0	25,200	1,600	6.4
1.5	33,800	11,500	34.0
3	40,350	550	1.4
6	297,000	320	0.11
9	730,000	140	0.02
12	1,350,000	540	0.04
15	2,730,000	5,120	0.19
18	3,410,000	58,000	1.7
21	3,000,000	55,000	1.8
24	3,170,000	71,800	2.3
27	3,240,000	83,000	2.6
30	2,710,000	129,000	4.8
33	2,560,000	184,000	7.2
36	3,200,000	184,000	5.8

maximum stationary phase. Because of the large number of samples to be heat shocked and plated at one time, triplicate tubes were inoculated from the respective inoculating cultures at varying intervals. The inoculating cultures were grown at the temperature to be used in the experiment. The results contained in tables 3 and 4 and figures 1 and 2 reveal certain changes in heat resistance undergone by *E. coli* as it passes through the various growth phases. The heat resistance at the 0 hour is naturally that of the inoculating culture. When active reproduc-

tion commences, the heat resistance decreases and according to the actual numbers of survivors shown in the table, it would seem that the survivors are cells which had not reproduced in the culture. As the rate of reproduction diminishes, there is a corre-

TABLE 4

Heat resistance of cultures of Escherichia coli grown at 38.5°C. for varying periods and then heat shocked at 53°C. for thirty minutes

AGE OF CULTURE	PLATE COUNT		PERCENTAGE SURVIVAL
	Before heating	After heating	
hours	*nos. per cc.*	*nos. per cc.*	
0	25,170	18,500	74
1	32,300	5,450	17
2	78,700	1,000	1.3
3	233,000	6,700	2.9
4	617,000	3,300	0.53
5	990,000	3,300	0.33
6	1,365,000	3,300	0.24
7	2,113,000	3,300	0.15
8	2,540,000	6,700	0.27
9	3,150,000	60,000	1.9
10	2,910,000	53,000	1.8
11	2,820,000	140,000	5.0
12	2,466,000	290,000	12
13	2,680,000	460,000	17
14	2,780,000	540,000	19
15	2,740,000	1,260,000	46
16	2,514,000	1,160,000	46
17	2,114,000	1,380,000	65
18	2,900,000	1,520,000	52
19	2,660,000	2,120,000	80
20	2,320,000	1,340,000	58
21	2,780,000	1,780,000	64
22	2,840,000	1,720,000	61
23	2,880,000	1,993,000	69
24	3,360,000	1,880,000	56

sponding rise in heat resistance until a peak is attained well along in the maximum stationary phase of growth. There follows a period which varies with the incubation temperature, during which period both the numbers of organisms and the heat resistance remain fairly constant, and after which there is a slow

and gradual decline in numbers and in resistance of the cells. Again the heat resistance of the 38.5° culture is far greater than that of the 28° culture. The one and one-half hour sample at 28° indicates an abrupt and brief increase in heat resistance comparable to that reported by Anderson and Meanwell (1936) for a thermoduric streptococcus.

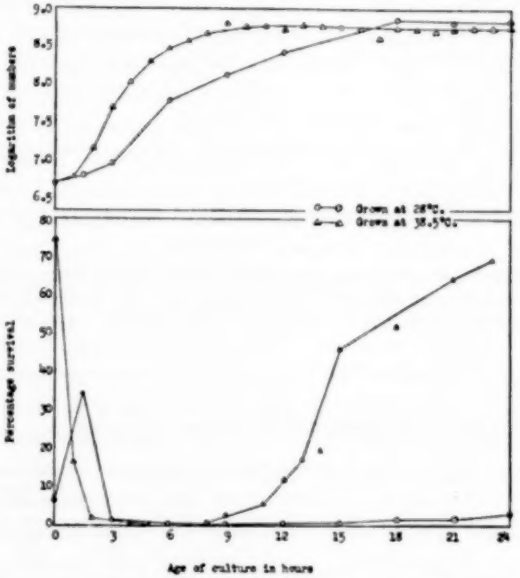

FIG. 1. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival of cells during heat treatment at 53°C. for thirty minutes. Age of cultures, 0 to 24 hours.

Heat resistance of cultures of E. coli during the initial stationary growth phase

The observation that there was an increase in resistance during the early hours of incubation at 28°C. led to a more thorough investigation of the heat resistance of *E. coli* during the first few hours of growth at 28° and at 38.5°C. Inoculating cultures, prepared from stock cultures, were incubated, respectively, at

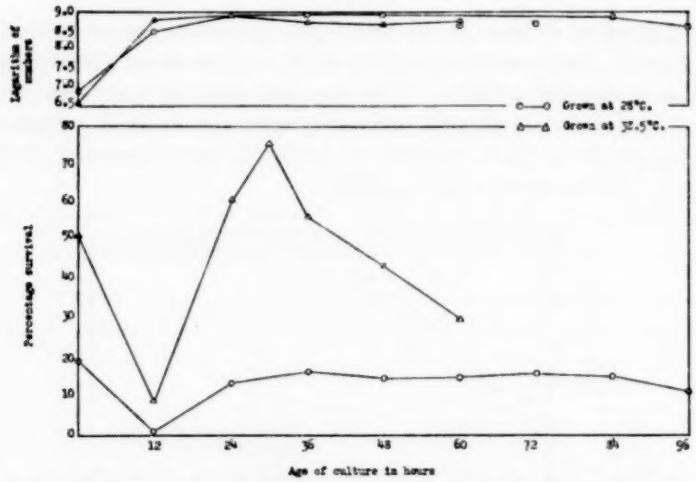

FIG. 2. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival during heat treatment at 53°C. for thirty minutes. Age of cultures, 0 to 96 hours.

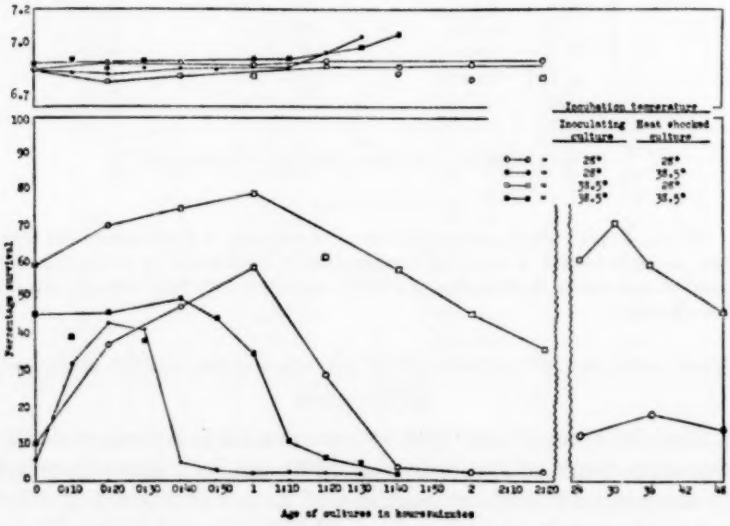

FIG. 3. Upper part shows growth curves of cultures of *Escherichia coli*; lower part shows influence of time and temperature of incubation on percentage survival of cells during heat treatment at 53°C. for thirty minutes.

28° for 48 hours and at 38.5°C. for 24 hours. Triplicate tubes were inoculated from these at varying intervals and incubated at 28° and 38.5°C., respectively. To determine the heat resistance of the tube cultures during the first three hours, the percentage survival was calculated. The results are contained in figure 3. The highest percentage survival obtained during the maximum stationary phase of growth at 28° and 38.5°C., respectively, is also shown for purposes of comparison.

Contrary to expectations, it was found that just before or during the "period of physiological youth" there was a distinct, abrupt rise in heat resistance of the culture, and then, a short time before reproduction commenced, a rapid decline in resistance to the low level characteristic of the phase of logarithmic growth.

However, the maximum percentage survival during the initial stationary phase of a culture inoculated from one grown at 38.5°C., generally exceeded that obtained with the subculture from the 28°C. inoculating culture. The greatest thermal resistance was demonstrated by 28° cultures inoculated from a 38.5°C. inoculating culture. When a 38.5°C. inoculating culture was transferred to fresh media with incubation at 38.5°C., no increase in heat resistance occurred during the lag phase of this subculture. These results, therefore, suggest that apparently some factor inherent in the inoculum plays an important rôle in determining the degree of heat resistance during the lag phase of growth.

DISCUSSION

It has been a common belief that a culture of bacteria, in any but the logarithmic growth phase, when placed in a medium advantageous for growth, passed through a stationary or lag phase and underwent a process of "biological rejuvenescence" which was followed by active reproduction. It has been believed that the heat resistance of the culture was notably low during the period of physiological youth and remained low as long as reproduction took place at a rapid rate. It is now apparent that still another change is manifest during the early life of a culture, and that this transitory change is characterized by a very decided increase in heat resistance. This increase is apparently more

marked when the culture is placed at a temperature below the optimum for growth.

In these experiments, it was found that during the period of most active reproduction at both 28° and 38.5°C. the heat resistance declined to its lowest point. Then as the cultures entered the maximum stationary phase, their heat resistance again rose. At 28°, however, the maximum percentage survival rarely reached more than 20 per cent and was usually less than 10 per cent, while at 38.5°C., 50 to 80 per cent was usually reached. At 30° the percentage survival was generally about 25 to 30 per cent. The maximum resistance at 40° and 42° was similar to that at 38.5°C. At the higher temperatures, therefore, the maximum percentage survival during the maximum stationary phase in every case exceeded that obtained at the temperatures below the optimum for growth. There are two possible explanations for such an effect of incubation temperature on heat resistance. The temperature at which the organisms are grown may in some manner influence the physico-chemical properties of the cells and thus render them more or less resistant to heat. The fact that the difference between heat resistance of 28° and 38.5° cultures was evident on the first transfer at the two temperatures would tend to discount this theory. Another explanation might be the following: At 38.5°C. reproduction and accompanying changes take place at about twice the rate at 28°C. Therefore, a greater accumulation of mature cells may be possible at 38.5° than could take place at any one time at 28°C. As a result, the maximum percentage survival during the maximum stationary phase would be decidedly greater at the higher incubation temperature.

In addition to their interest from the standpoint of bacterial physiology, the results reported above are of significance for various other reasons. *E. coli* has long been used to determine the efficiency of different methods of destroying vegetative cells. It is apparent that the resistance of *E. coli* to heat and possibly to other adverse factors varies markedly with the time and temperature of incubation and that these, together with the medium in which the organisms are grown before or following heat or

other exposure, will play an important part in determining the number of surviving cells.

As indicated by Anderson and Meanwell, organisms other than *E. coli* may increase decidedly in heat resistance during the early hours of growth. Therefore, the temperature at which milk is held previous to pasteurization may profoundly influence the efficiency of the process. The survival of organisms during other processing treatments, intended primarily to decrease the number of vegetative cells in a liquid, may be affected in the same manner.

It has been shown by Elliker (1937) that the culture medium and time and temperature of incubation might have a significant effect on the activity of Swiss cheese starter cultures. Lactobacilli growing more rapidly in a favorable medium generally demonstrated greater heat resistance than did those growing more slowly in a poor medium. Furthermore, when the same organisms were grown in a poor medium, a slight elevation of the incubation temperature and, therefore, a consequent increase in rate of growth usually resulted in a more heat resistant culture. In the present studies with *E. coli*, greatest heat resistance during the maximum stationary phase was exhibited at the higher temperatures where rate of growth had been comparatively rapid.

The results of Anderson and Meanwell and the observations reported in this paper indicate that starter cultures may also exhibit an increase in resistance during the initial stationary phase of growth and that this increased thermal resistance may significantly influence the behavior of the starter organisms during the early stages of the cheese making process, particularly if the cheese be one like Swiss where comparatively high temperatures are employed in the making process.

Future investigations may determine whether or not other species of bacteria demonstrate the same changes in thermal resistance as do the thermoduric streptococcus used by Anderson and Meanwell and *Escherichia coli*.

SUMMARY

Cultures of *Escherichia coli* exhibited a decided increase in heat resistance, as evidenced by percentage survival of cells during

heat treatment, while in the initial stationary phase of growth. The increase in heat resistance was more marked in cultures incubated at 28° than in those incubated at 38.5°C. The time and temperature of incubation of the culture used for inoculum decidedly influenced the degree of increase in heat resistance during the initial stationary phase of growth of the subculture.

The heat resistance of all of the cultures decreased as reproduction commenced and their resistance fell to a minimum during the period of most active reproduction. The resistance then increased again to a second peak as the rate of reproduction decreased and the culture entered the maximum stationary phase of growth.

Growth at and above the optimum temperature resulted in cultures whose heat resistance during the maximum stationary phase was distinctly greater than was true of cultures incubated at temperatures below the optimum for growth.

Possible reasons for the variations between heat resistance of cultures grown at high and low temperatures and the practical significance of the results are discussed.

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BOUND WATER CONTENT OF VEGETATIVE AND SPORE FORMS OF BACTERIA

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In a previous paper (Henry and Friedman, 1937) we confirmed the works of Dyrmont (1886) and Virtanen and Pulkki (1933) which showed that little difference exists in the water content of vegetative cells and spores of a given species of bacteria. These results indicated that the commonly accepted idea that a low water content in the spore form is responsible for the observed heat resistance of this type of cell was not justified.

Virtanen and Pulkki advanced the theory that the enzymes present in the bacterial spore were in an inactive or resistant form. We suggested that the resistance, whether it concerned the enzymes or the bacterial protoplasm proper, might be due to differences in the percentage of bound water in the two types of cells. This suggestion was based on the report of Newton and Martin (1930) which shows that the resistance of certain plants to drought and freezing is, in part, due to their relatively high percentages of bound water.

The present paper is a report of the relative amount of bound water found in the vegetative cells and spores of *Bacillus mycoides*, *Bacillus megatherium* and *Bacillus subtilis*, as determined by the cryoscopic method (Newton and Gortner, 1922). This method was chosen because of its relative simplicity and because of the similarity of our problem to that of Skovholt and Bailey (1935) when they determined the bound water in flour. The procedure is based on the assumption that bound water does not alter the freezing point of a given solution of sucrose, and therefore if a weighed quantity of bacterial cells with a known water

content, as determined by desiccation of a portion of a uniform sample, is added to a sugar solution, changes in the freezing point of the solution will be due to the unbound water; the difference between the amount of water which affects the freezing point of the solution and the total water content as determined by desiccation should represent water in a bound state. While the method employed is probably not sufficiently accurate to make the percentages of bound and free water found in a given cell suspension entirely reliable, it is possible to show that these errors will be similar in two determinations when conditions are controlled and that comparative data of significance may be obtained when suspensions of two types of cells are run under identical conditions.

METHODS

Suspensions of vegetative cells of *B. subtilis*, *B. mycoides* and *B. megatherium* were procured by growing the organisms on a medium of the following composition:

	grams
Glucose	2.5
Peptone	5.0
Sodium chloride	0.5
Dipotassium phosphate	5.0
Ammonium sulphate	2.5
Water	1000 ml.

Spores were obtained on a medium identical with that given above, except for the addition of 1.75 per cent agar. After harvesting, the cells were washed four times in distilled water and examined microscopically to determine the ratio of spore to vegetative cells. In all suspensions used the ratio of the desired type of cell to the other form was 200 to 1 or higher.

After thorough washing, the bacteria were blotted between silk and filter paper and prepared for sampling by being well mixed on a silk cloth. Representative samples were transferred to dry, weighed containers and one of these used to determine the water content while the others were used to measure the effect upon the freezing point of water or a standard sucrose solution. The

total water content of the cells was determined by heating at 100–105°C. until a constant weight was obtained. This latter procedure was checked several times and shown to give consistent results.

EFFECT OF BACTERIA UPON THE FREEZING POINT OF DISTILLED WATER

By the addition of various quantities of a single mass of wet, vegetative cells of *B. mycoides*, the water content of which was determined, to known quantities of distilled water the effect of several concentrations of the cells themselves upon the lowering of the freezing point was obtained. After correcting for undercooling by using the tables of Harris (1925) it was possible to calculate the true freezing points of these suspensions on the basis of grams of solids per 100 grams of water. The results obtained are as follows:

BACTERIA	WATER	FREEZING POINT
grams	*grams*	°C.
0.6627	100	−0.018
1.1803	100	−0.022
3.1705	100	−0.030

If these points are plotted, using lowering of the freezing point against grams of organisms per 100 grams of water, a straight line relationship can be demonstrated. Because of this linear relationship the effect of any quantity of bacterial material upon the freezing point of water can be obtained.

DETERMINATION OF BOUND WATER IN BACTERIAL CELLS

Accurately weighed quantities of moist bacteria with a known water content were placed in distilled water and in sucrose solution. This sugar solution was prepared by adding 16.000 grams of sucrose, which had been dried three days over fresh calcium chloride, to sufficient water to make 100 ml. of solution. Density measurements by the pycnometer method were made and the concentration of sucrose was found to be 0.1507(5) gram for each gram of solution used. The weights of total water, bac-

terial solids and sucrose were determined and the freezing point for each mixture was established. Masses of spores and vegetative cells of a given species were run in the same manner and all solutions and procedures were as nearly identical as possible for the two types of cells.

As an example of the results thus obtained the figures for *B. subtilis* are given in table 1.

TABLE 1

The effects of B. subtilis spores and vegetative cells upon the freezing points of distilled water and sucrose solution

	VEGETATIVE CELLS 68.86 PER CENT WATER, 31.14 PER CENT SOLIDS		SPORES 72.38 PER CENT WATER, 27.62 PER CENT SOLIDS	
	Distilled water	Sucrose solution	Distilled water	Sucrose solution
Moist weight of bacteria	0.3693	0.4609	0.5072	0.5581
Weight water added	13.0532		14.0895	
Weight sugar solution added		14.6864		15.1076
Freezing point	−0.019	−1.034	−0.020	−1.066
Undercooling, degrees C	1.0	0.8	0.6	1.1

From these data, again using *B. subtilis* as an example, it is possible to obtain or calculate:

1. The dry weight of the bacteria in sugar solution:
   Vegetative cells ..... 0.1435 gram
   Spores ..... 0.1541 gram
2. The weight of sucrose in the sugar solution:
   Vegetative cells ..... 2.2139 grams
   Spores ..... 2.2775 grams
3. The total weight of water in the sugar solution:
   Vegetative cells ..... 12.7898 grams
   Spores ..... 13.2340 grams
4. The weight of organisms per 100 grams of water:
   Vegetative cells ..... 1.122 grams
   Spores ..... 1.165 grams
5. The corrected freezing points in sugar solution:
   Vegetative cells ..... −1.028°C.
   Spores ..... −1.051°C.
6. The corrected freezing points in distilled water for quantities of bacteria equal to those used in sugar solutions:
   Vegetative cells ..... −0.024°C.
   Spores ..... −0.022°C.

7. The weight of sucrose per 100 grams water:

Vegetative cells	17.3098 grams
Spores	17.2094 grams

By applying the formula developed by Gortner and his coworkers (Newton and Gortner, 1922) to these figures, it is possible to calculate the bound water present in the system. This formula is:

$$\frac{T-(t+K)C}{T-t} = \text{per cent bound water.}$$

where T = observed lowering of freezing point in sugar solution containing bacteria.

t = lowering of freezing point in distilled water plus bacteria (corrected).

K = constant calculated for lowering of freezing point of sucrose solution.

C = constant dependent upon concentration, related to amount of available water.

For first calculations the value of K was that of Sayre's (1932). The value of C may be calculated:

$$100 - \frac{\text{(grams sucrose per 100 grams water) (18) (6)}}{342.2}$$

The percentage of bound water multiplied by the weight of the total water in the system gives the weight of bound water. In the following calculation the change in C due to water added with the mass of organisms is disregarded as insignificant.

Results obtained by this treatment of the data gave, in some cases, a negative value and for this reason a constant K^1 was used. K^1 was obtained by assuming 0.014° less lowering, for all concentrations of sugar, than the values observed by Sayre. This procedure is justified in that our interest lies in comparative rather than absolute values for the amount of bound water present in the various cell suspensions. Also as Sayre says: "This difference may be due to some systematic error in all freezing point measurements, such as purity of sucrose used, the degree of undercooling, or the calibration of the thermometer."

By similar treatment of data obtained when the vegetative

and spore forms of *B. megatherium* and *B. mycoides* were used the water binding capacity of bacterial cell materials of the three species was determined. Table 2 gives results expressed as grams of water bound per gram of solids and also the per cent of bound water in the moist bacterial masses. These latter figures were obtained in the following manner:

(Grams solids per 100 grams moist mass)
(grams water bound per gram solid) = per cent bound water.

If the amount of water which may be bound by the solids in a given cell mass is subtracted from the total water present, the percentage of free water in *B. subtilis* vegetative cells would be

TABLE 2

The bound water content of vegetative cells and spores of three bacterial species

	B. SUBTILIS		B. MEGATHERIUM		B. MYCOIDES	
	Vegetative	Spores	Vegetative	Spores	Vegetative	Spores
Grams bound water per gram solids	0	2.5	0.8	1.9	1.3	2.0
Per cent bound water in bacterial mass	0	69.0	17.7	62.6	28.2	58.7

68.9, in spores 3.4; for *B. megatherium*, 60.2 and 4.5, and for *B. mycoides* 50.0 and 11.9.

The thermal death time at 100°C. was determined for suspensions of spores of these three species which had been grown on the medium described above.

That a rather close correlation was found between the calculated free water and the relative heat resistance of the spores is shown below:

	FREE WATER	THERMAL DEATH TIME AT 100°C.
	per cent	*minutes*
B. subtilis spores	3.4	6
B. megatherium spores	4.6	4
B. mycoides spores	11.6	2

The free water, as determined by us, approximates the total water content which in the past has been assumed for spores in explaining their heat resistance on this basis.

Thermal death point determinations showed that the vegetative cells of all three species are destroyed at approximately the same temperature (50°C.). This would be expected if the work of Lewith (1890) on the effect of various concentrations of water on the coagulation temperature of albumin is accepted. Lewith showed that variations in high water concentrations did not materially affect the coagulation temperature of egg albumin, whereas low concentration differences markedly influenced the coagulation temperature.

SUMMARY

1. Bound water determinations, by the cryoscopic method, have been made on the vegetative and spore forms of *Bacillus subtilis*, *Bacillus megatherium* and *Bacillus mycoides*.

2. In all cases the spores were shown to have a far greater water binding capacity than did the vegetative cells.

3. The theory is advanced that the heat resistance of bacterial spores is due in part at least to the relatively high percentage of water in the bound state.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

EASTERN MISSOURI BRANCH

SPRING MEETING, ST. LOUIS, APRIL 12, 1938

ATTEMPT TO SECURE FERMENTING VARIANTS BY SERIAL TRANSFERS IN SUCROSE. *MacDonald Fulton*, Saint Louis University School of Medicine, St. Louis.

Sucrose-fermenting variants failed to appear when a group of 30 strains representing 8 species of sucrose-negative *Escherichia* were transferred serially in sucrose broth according to the method of Tregoning and Poe (Jour. Bact. 1937, *34*, 465). Storage for 6 weeks at 37° and subsequent plating also failed to reveal fermenting variants. The statistical significance of these opposing results was examined and their bearing on the theory of dissociation discussed.

SOME EFFECTS OF CARCINOGENIC SUBSTANCES ON YEASTS. *C. W. Dodge*, Missouri Botanical Garden, St. Louis.

NOTES ON THE DIAGNOSIS AND TREATMENT OF TULAREMIA. *George A. Hunt and Wm. F. Friedewald*, City Hospital No. 1, St. Louis.

The intradermal injection of a formaldehyde-killed, saline suspension of *Pasteurella tularense*, together with the agglutination reaction and the opsonocytophagic test proved most useful for an early diagnosis of tularemia. The use of an intradermal injection of a specific antiserum, described by Foshay, as a diagnostic aid, deserves further study since a positive reaction with goat antitularense serum and a negative reaction with normal goat serum was always confirmed by other diagnostic tests. It is difficult, however, to obtain animals for immunization whose sera do not react with many normal individuals.

A modification of the opsonocytophagic test, which may be of some value to State diagnostic laboratories finding it difficult to obtain fresh blood specimens, consists of the use of equal volumes of fresh citrated normal blood, the serum to be examined, and a suspension of *Pasteurella tularense* or *Brucella* organisms.

In a study of fifty cases of tularemia over a period of two years (thirteen cases having been given serum within the first three weeks of the disease), the observations on treatment agree essentially with those made by Foshay.

A MANOMETRIC METHOD FOR THE EVALUATION OF ANTISEPTICS. *J. Bronfenbrenner, A. D. Hershey and J. A. Doubly*, Washington University School of Medicine, St. Louis.

The parasitotropic and organotropic properties of antiseptics were determined in the Warburg-Barcroft apparatus. Measurements were made of the effect of antiseptics upon the rate of oxygen uptake by *Escherichia coli* and adult mouse liver tissue respectively, as compared to the rate of oxy-

gen uptake in absence of antiseptics as control.

The concentration of antiseptics which causes a 50 per cent reduction in the oxygen uptake in a given time interval is taken as an arbitrary end point. The results obtained with *E. coli* show that for the antiseptic investigated, the effective concentrations fall approximately half way between the final bacteriostatic and germicidal concentrations as determined by the test tube culture method.

For purposes of comparison it is possible to calculate a respirometer coefficient in terms of phenol or bichloride of mercury.

A toxicity index for each antiseptic can be calculated from the results obtained on liver tissue and *E. coli*. The relation between the two values gives an indication as to the therapeutic possibilities of the compound. The values so determined are being compared at present with the effectiveness of the corresponding compounds in *in vivo* tests. To date our experience has been limited to two test materials (mouse liver cells and *E. coli*). We hope, however, to extend this study to other tissues and organisms.

The method has an advantage in that the results are immediately available.

A Suggested Laboratory Method for Testing Fungicides Recommended for the Treatment of Epidermophytosis. *E. Burlingame, and G. F. Reddish*, Lambert Pharmacal Co., St. Louis.

The following organisms are used: *Epidermophyton inguinale, Epidermophyton intergitale, Epidermophyton rubeum, Epidermophyton gypseum, Trichophyton rosaceum.* Each organism is streaked over the surface of Sabouraud's agar in a 9 cm. Petri dish, using a dry sterile cotton swab inoculated with a five-day culture of these organisms. These plates are incubated at room temperature for five days. The agar cultures are then cut into 1 cm. squares. The fungicide to be tested is then poured over the surface of the culture so as to entirely flood the plate. After 5, 15 and 30 minutes one of the squares of culture and agar is removed and placed into 10 cc. of sterile broth. The excess fungicide is washed out of the matted culture by shaking the tube lightly for two minutes. At the end of this time the block of culture is removed from the broth and streaked with the culture side down over the surface of a sterile plate of Sabouraud's agar. These plates are then incubated at room temperature for three weeks and observed for growth.

An effective fungicide should kill these test organisms within 5 minutes. Experience has shown that fungicides which kill the test organisms in 5 minutes by this test are effective in the treatment of epidermophytosis as determined by clinical test. It has also been proved that preparations which do not kill these organisms by this test within 30 minutes are not effective under clinical conditions. The time period selected should be between these two points and 5 minutes is suggested.

This test is simple, requires very little material, makes use of the organisms which are the most common cause of "Athlete's Foot", makes use of solid medium in which these organisms grow most luxuriantly, uses a large inoculum, and avoids satisfactorily any bacteriostatic activity of the fungicides tested. This method has been carefully checked by extensive clinical tests and found to be satisfac-

tory for the purpose of indicating which fungicides will be effective under practical conditions of use.

THE QUEST OF THE ALASKA SEALSKIN. *Helen Norris Moore*, Fouke Fur Co., St. Louis.

A moving picture in color was shown depicting the annual expedition to the Pribilof Islands to take the U. S. Government sealskins. Scenes were shown of the flora of the islands, the life and habits of the fur seals, and the methods used in preparing the pelts for shipment to St. Louis for dressing and dyeing.

The bacteriological problems which were discussed included studies of salt tolerant organisms, effects of germicides, and routine counts of various liquors used during the process of preparing these furs.